

THE IMMUNOLOGICAL POTENTIALITIES
OF THE LYMPHOCYTES OF HOMOGRAFT TOLERANT RATS

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STATEMENT

The experiments recorded in this thesis were done by myself.

Mr R. Hill prepared and cut the histological sections.

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ABSTRACT

The present experiments were intended to examine the nature of the cellular responses involved in the termination of homograft tolerance. The transfer of normal syngeneic lymphocytes terminated tolerance, as evidenced by the destruction of hitherto tolerated skin grafts. Second skin grafts placed on such "terminated-tolerant" rats at any time after termination of homograft tolerance were rejected as rapidly as grafts placed on normal rats. However, the graft-versus-host activity of lymphoid cells from these "terminated-tolerant" rats remained remarkably low, in comparison with that of cells from normal rats, over a prolonged period. The graft-versus-host reactivity of lymphoid cells from "terminated-tolerant" rats eventually returned to a normal level after the rejection of skin grafts.

Experiments in which the passage of lymphocytes with the capacity to terminate tolerance through a series of tolerant hosts was attempted implied that the elimination of chimaeric donor cells from the tolerant animal, coincident with skin graft rejection, may be the major factor determining the subsequent reappearance of immunological normality. In the absence of graft rejection reactive cells fail to reappear in the lymphoid tissues of a tolerant recipient. The return of a previously tolerant animal to immunological normality was demonstrated to be a consequence of the reappearance of reactive host cells rather than of the proliferation of the transferred normal lymphocytes. The extended period which was required for the full return of immunological normality was more consistent with the generation of reactive cells by the expansion of a small population of precursors than with their recruitment as a result of the reactivation of reversibly suppressed cells.

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ABBREVIATIONS

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B. Factors influencing the induction of tolerance

1) Induction of tolerance by means of living cells

T.D.L. thoracic duct lymphocytes

a) Genetic relationships between donor and

SP. recipient spleen cells

b) Immunological state in embryo and neonatal

L.N.C. lymph node cells

c) Antigen dose

B.M.C. bone marrow cells

a) The age of the recipient when induction of

G.v.H. graft-versus-host

b) Induction of tolerance in the adult

F₁ F₁ hybrid

i) Weak histocompatibility barrier

ii) Strong histocompatibility barrier

No. Number.

iii) Induction of tolerance in prenatally

adult recipients

2) Induction of tolerance by means of non-living

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SECTION 1

Review of the Literature

A. The early history of homograft tolerance

Investigation of the phenomenon of immunological tolerance commenced with the observation by Owen (1945) that chimaerism could occur in calves which had shared circulations in utero. This was established by demonstrating that each animal possessed two hematopoietic cell populations, one of which was derived from the non-identical twin. A few years later, Burnet and Fenner (1949) emphasized the wide significance of Owen's observation in proposing a general theory of the immune response that predicted, among its consequences, the phenomenon of tolerance. To explain the failure of an adult animal to react immunologically against its own tissues, either in their normal state or as damaged body constituents, these authors suggested that its cells possessed some type of "self-marker" component, the capacity to recognize which was absent during the early stages of development. As an extension of this hypothesis, they predicted that exposure to antigen in embryonic life would result in that antigen being recognized as "self" in later life as a consequence of which no immune response would be mounted against it. Further studies showed that most dizygotic cattle twins would accept skin grafts from each other and that this mutual tolerance was specific in that skin transplanted from third parties was quickly rejected (Anderson, Billingham, Lampkin and Medawar, 1951; Billingham,

Lampkin, Medawar and Williams, 1952). This series of experiments on "natural embryonic parabiosis" and Burnet and Fenner's prediction stimulated both Medawar's and Hasek's laboratories to attempt the experimental induction of tolerance in laboratory animals.

Although tolerance of skin homografts has been induced in mice, rats, rabbits and chickens, most of the basic work has been carried out in mice because of the availability of highly inbred strains. The initial and subsequent experiments by Billingham, Brent and Medawar (1953, 1956a) to induce tolerance involved the injection of mouse embryos of the CBA strain in utero with suspensions of living spleen cells from mice of the A strain. When these CBA recipients attained adult life, they were regularly found to accept A-strain skin grafts. The tolerance so induced was specific since CBA mice tolerant of A strain tissues easily rejected allografts from the unrelated AU strain.

Tolerance was induced in the chicken by the intravenous injection of 10 day old embryos (Billingham, Brent and Medawar, 1953), but Hasek's ingenious technique of embryonic parabiosis gave even better results. Parabiosis facilitated a prolonged mutual exchange of embryonic blood cells, and a very high degree of tolerance of each other's skin grafts was established in the parabionts (Hasek, 1953a, 1953b). The special value of parabiosis of avian embryos was its efficacy in establishing not only tolerance of skin grafts, but also a long-lasting red cell chimaerism which could be demonstrated by conventional serological tests (Billingham, Brent and

Medawar, 1956a).

Subsequently, it was demonstrated that in utero injection was not necessary and that mice injected intravenously within the first few days after birth could be rendered tolerant (Woodruff and Simpson, 1955; Billingham and Brent, 1957). In order to explain these observations, Burnet proposed, in his clonal selection theory (Burnet, 1959), that the interaction of an antigenic determinant with a cell which possesses a genetically determined affinity for that antigen will result in the death and elimination of that cell, provided that this interaction occurs during the embryonic or perinatal period. Hence, no immune response would occur against self-components, because all cell clones potentially responsive to self determinants would have been destroyed during embryonic life. If tolerance depends upon the elimination of a specific cell population (Burnet, 1957, 1959), Burnet suggested that the occurrence of autoimmunity or "escape" from tolerance could result from a mutation from which a "forbidden" cell clone could develop (Burnet, 1962) or from the resumption of cellular differentiation.

While the major contribution which the clonal selection theory made to the comprehension of immunological tolerance refers to self-tolerance, the theory has been generally accepted as an explanation for artificially induced forms of tolerance. Similarly, by extrapolation, it has been inferred that the termination of the tolerant state to non-self antigens would require the appearance or provision of new cells with the appropriate reactivity.

B. Factors influencing the induction of tolerance

1) Induction of tolerance by means of living cells

a) Genetic relationships between donor and recipient

On account of the requirement for inbred animals in quantitative tolerance experiments, most of the basic work has been carried out in mice and rats. It is well established that the difficulty in inducing tolerance between unrelated animals increases with genetic disparity between donor and recipient. Such a situation occurs in mice when donor and recipient differ at the strong H-2 histocompatibility locus and in rats when the difference is at the Ag-B locus. When such a strong genetic barrier is involved larger inocula of cells are required, the intravenous route of injection is almost obligatory, and the tolerance-responsive period extends for only a short time after birth. On the other hand, if the genetic disparity involved is relatively weak, such stringent requirements do not apply.

b) Immunological state in embryo and neonatal animals

It was initially believed that tolerance could only be induced at a time before the animal had developed immunological responsiveness, that is before it was capable of reacting against the inoculum itself. However, it was found that, whereas large doses of allogeneic spleen cells injected neonatally did indeed produce tolerance, very small doses could induce sensitivity. This sensitivity was detected by an ingenious assay based on the protective effect conferred

by the inoculum against a subsequent graft-versus-host reaction (Howard and Michie, 1962, 1963; Howard, Michie and Woodruff, 1962).

Further evidence for the presence of immunologically competent cells in newborn mice was presented by Brent and Gowland (1963a), who noted that the prior injection of a small dose of splenic cells decreased, to some extent, the tolerant state induced by a subsequent, large weight-adjusted dose of such cells. These authors concluded that immunologically competent cells are present in the neonate, but that they occur in much smaller numbers than in later life. It should be emphasized that evidence also exists that foetal mammals of other species can initiate immunologic responses (Schinkel and Ferguson, 1953; Uhr, 1960; Uhr, Dancis and Neumann, 1960; Silverstein, Prendergast and Kraner, 1964).

c) Antigen dose

Brent and Gowland (1962a, 1962b, 1963b), and Gowland (1965) using both H-2 compatible and H-2 incompatible strain combinations of donor and newborn recipient, clearly showed that it is approximately 20 times more difficult to overcome a strong (H-2) than it is to overcome a relatively weak (H-1, H-3) genetic barrier. Similar results have been obtained in mice by Billingham and Silvers (1962).

Silvers and Billingham also showed the strong influence of the Ag-B locus on tolerance induction in rats (Silvers and Billingham, 1969a). For example, 70% of adult Lewis rats inoculated at birth with 4×10^7 bone marrow cells from Ag-B incompatible BN rats displayed a high degree of

tolerance, whereas when the neonatal injection was derived from Fischer rats (Ag-B compatible) as few as 10^6 marrow cells induced a high degree of tolerance of Fischer grafts in about 90% of the Lewis subjects.

d) Source of antigen

Variation in the tolerance-inducing capacities of cells derived from different components of the lymphohematopoietic tissues in mice and rats was demonstrated by Billingham and Silvers (1961a, 1962). Furthermore, a striking difference was revealed between the tolerance-responsiveness of neonatal mice and rats when a comparison was made between the tolerance-inducing capacity of cells derived from different tissues. In the mouse, a detailed analysis using an H-2 locus incompatible strain combination (CBA and A) revealed that allogeneic lymph node cells and splenic cells were much more effective than bone marrow cells in rendering newborn recipients tolerant of skin homografts, though lymph node cells were much less effective when semi-allogeneic F_1 hybrid strain cells were used. However, in the rat, it was found, using an Ag-B incompatible strain combination, that bone marrow cells were vastly superior to both lymph node and spleen cells in inducing tolerance of skin grafts from Ag-B incompatible donors, cells from these latter tissues proving to be only marginally effective in inducing high degrees of tolerance.

The greatest disparity in efficacy as tolerogenic inocula for Ag-B incompatible and Ag-B compatible recipients occurred with thymocytes (Billingham and Silvers, 1964). With

the BN \rightarrow Lewis combination high degrees of tolerance have never been obtained, even with inocula of 6×10^7 thymocytes. However, all Lewis rats injected neonatally with 10^7 Fischer strain thymocytes from adult donors subsequently accepted test homografts of Fischer strain skin for longer than 50 days (Silvers and Billingham, 1966, 1969a).

e) The age of the recipient when induction of tolerance is attempted

The effect of the intravenous injection of (CBA \times A) F_1 hybrid strain spleen cells in inducing tolerance in neonatal A-strain recipients of various ages has been examined. It was found that, if a constant dose of cells is administered to each animal, the percentage of mice rendered highly tolerant declined rapidly with the age at which they were injected (Brent and Gowland, 1961, 1962b). If the dose of spleen cells is adjusted to compensate for the increase in body weight of the recipients with age, the frequency with which highly tolerant animals are produced in each age-group is increased. However, the period during which tolerance can be induced is extended for only a week by this manoeuvre. A weight-adjusted dose of lymphoid cells failed to induce tolerance in any of the 8 day old recipients. Furthermore, a single injection of ten times the weight-adjusted dose for 13 day-old mice failed to induce tolerance in such animals.

f) Induction of tolerance in the adult

i) Weak histocompatibility barrier. It is well documented that tolerance of tissue allografts can be induced in adult mice in certain strain combinations, which differ

only at weak histocompatibility loci. Parabiotic union between allogeneic individuals (Rubin, 1959; Martinez, Shapiro, Kelman, Onstad and Good, 1960; Jensen and Simonsen, 1962), the intravenous administration of a single large dose of viable splenic cells (Mariani, Martinez, Smith and Good, 1959; Shapiro, Martinez and Good, 1959), and the repeated injection of splenic cells intravenously or intraperitoneally (Shapiro, Martinez, Smith and Good, 1961) have all been found to be effective. In general, the induction of tolerance in immunologically mature animals requires their exposure to very large doses of antigen, irrespective of the method of exposure.

ii) Strong histocompatibility barrier. The induction of tolerance across strong incompatibilities presents a formidable problem. The induction of tolerance has been reported in the (C3H x A) $F_1 \rightarrow$ C3H strain combination, which differs at the weakest H-2 locus, by means of only one intravenous injection of spleen cells obtained from F_1 hybrids of the two strains involved (Guttman and Aust, 1961). Similarly, repeated injections of large doses of antigen over prolonged periods of time (up to 7 weeks) have been successful in inducing tolerance in H-2 incompatible (C3H x A) $F_1 \rightarrow$ C3H mouse strain combinations (Shapiro, Martinez, Smith and Good, 1961; Martinez, Shapiro and Good, 1962). However, in a similar experiment, Gowland (1965) failed to confirm the data obtained by Shapiro et al. (1961) and Martinez et al. (1962). It was shown that, in the combinations of (CBA x A) $F_1 \rightarrow$ A and (CBA x A) $F_1 \rightarrow$ CBA,

tolerance could not be obtained with cumulative doses in excess of those used by Shapiro et al. and Martinez et al. Tolerance was observed only when the weakest genetic difference was used, namely C3H \rightarrow CBA, and with a total dose of cells in excess of that found to be effective by Shapiro et al. and Martinez et al. in overcoming a strong (H-2) genetic barrier.

iii) Induction of tolerance in presensitized adult recipients. Gowland (1965) also showed that a regimen found to be effective in tolerance induction in H-2 compatible strains is totally ineffective in recipients previously specifically sensitized with donor antigens.

2) Induction of tolerance by means of non-living cells or cell extracts

Billingham and Silvers (1960) reported a significant prolongation of male skin graft survival on female mice of the C57BL strain treated at an early age by repeated injections of an antigenic extract prepared from lymphoid tissue of syngeneic male mice. Linder (1961) induced tolerance of male skin isografts in adult (DBA x C57BL) F_1 female mice using a cell-free preparation taken from the spleen, kidney and liver of male donors. Of 6 adult females receiving 12 intraperitoneal injections of this material over a period of 3 or 4 months, 5 became tolerant and accepted the corresponding male skin isografts permanently. Also Medawar (1963) reported the use of cell free preparations of lymphoid tissues to prolong homograft survival in adult mice receiving a single injection of the antigenic material combined with treatment by X-irradiation, amethopterin, or homologous isoantibody.

A further experiment in this series revealed that permanent tolerance of male skin isografts could be regularly produced in female mice of the C57BL strain during adult life by repeated injection of completely disrupted spleen cells derived from male donors (Martinez, Smith, Blaese and Good, 1963).

Tolerance was also induced in 3 of 11 DBA/2 mice during adult life by the repeated injection of completely disrupted spleen cells from Balb/c donors. In these experiments, DBA/2 mice (2 to 3 months of age) were given a total of 18 injections of spleen cell extract twice a week in a dose of 1 spleen equivalent per injection. After the 4th injection these animals were test-grafted with Balb/c skin, while the treatment was continued for an 14 additional injections. If only 4 or 5 additional injections were given after these animals were test-grafted, homograft tolerance of skin was not achieved in any animals, but prolonged survival of skin grafts could be produced in some recipients of the longer regime.

The surprising result that homograft tolerance across even the strong H-2 histocompatibility barrier could be obtained in the neonatal period and during adult life by the repeated injection of disrupted spleen cell preparations was also obtained in this experiment. For example, 2 out of 10 C3H mice prepared by repeated injection (18 times) during adult life of disrupted spleen cell preparation from (C3H x A) F_1 mice subsequently accepted skin grafts from (C3H x A) F_1 donors. Similarly, 1 out of 7 C3H mice which had received

22 injections of disrupted spleen cells from A strain mice became tolerant and accepted an A strain skin graft permanently. A further 4 out of 8 of C3H mice, so treated, accepted allogeneic grafts of a mammary adenocarcinoma which had developed spontaneously in an A strain host. Of particular importance was the finding that, when mice of the C3H strain received a series of injections of disrupted A strain spleen cells from birth until weaning, 4 out of 7 were found to be tolerant to A skin grafts.

3) Reduction of the immunological responsiveness of the adult to facilitate induction of tolerance

Suppression of the immunological responsiveness of adult animals by means of irradiation and immunosuppressive drugs greatly increases the efficiency of a given dose of antigen in inducing tolerance. It has been suggested that animals treated in this way come to resemble the newborn in respect of cell number or cell type in the lymphoid tissue (Schwartz and Dameshek, 1963). Sublethally irradiated adult mice could be rendered permanently tolerant of homografts from donors sharing the same H-2 allele, by the transfusion of allogeneic marrow or spleen cells (Fefer and Davis, 1963; Davis and Cole, 1963). Furthermore the successful induction of tolerance in adult mice across the H-2 barrier using sublethal irradiation and injection of donor spleen cells in high dosage has been reported by Michie and Woodruff (1962). Drug-induced immunological tolerance of homografts has been reported by Uphoff (1961) and McLaren (1961). However, it is doubtful whether the acceptance of skin homografts on

radiation chimaeras following lethal irradiation represents homograft tolerance. The death of such chimaeras, which had been produced by the injection of F_1 hybrid strain cells, following the transfer of lymph node cells from host origin implied that complete replacement of host tissue by donor cells had occurred (Cole and Garver, 1959).

Other forms of immunosuppressive treatment have been reported as facilitating the induction of tolerance in adult animals but have not been fully investigated. They include; the chronic drainage of lymphocytes from the thoracic duct (McGregor and Gowans, 1964), the administration of serum α_2 -globulin (Kamrin, 1959) and the administration of anti-lymphocyte serum (Woodruff and Anderson, 1963; Levey and Medawar, 1966).

C. The relationship of cellular chimaerism to the maintenance of tolerance

1) The occurrence of chimaerism in tolerant animals

Many investigations have attempted to correlate the immunological activity of tolerant animals with the extent of tissue chimaerism. In the case of animals rendered tolerant by neonatal injection of homologous cells, Billingham and Brent (1959) inferred that chimaerism was present after demonstrating that donor-strain antigen persisted. This persistence was established by injecting cells from adult A strain mice, which were tolerant as the result of neonatal injection of CBA spleen cells, into normal adult A strain mice. CBA strain skin grafts subsequently placed on these injected mice were subject to accelerated rejection indicating

sensitization to CBA specific antigens. Using this technique, chimaerism could be demonstrated in spleen, lymph node, thymus, bone marrow, kidneys, liver and blood. With a similar experimental protocol, Brent and Gowland (1963b) identified donor cells in the spleen of tolerant mice and estimated their frequency as being less than 5%. Significantly, donor type cells could not be detected in the spleens of mice in which the tolerant state had been abrogated as a result of the transfer of sensitized syngeneic cells 23 days previously.

Cellular chimaerism was also found (Michie, Woodruff and Zeiss, 1961) in homograft tolerant mice by means of Simonsen's discriminant spleen assay (1960). Adult survivors of mice which had been injected at birth with homologous spleen cells were uniformly tolerant of donor skin. A small percentage of chimaeric donor cells was demonstrable in these tolerant hosts because of their immunologic activity against a third strain of mouse. These results with the discriminant spleen assay were interpreted as indicating that a mutual accommodation was ultimately reached between donor and host components in the chimaera with each acquiring specific tolerance of the other.

The contrasting observation of Trentin and Session (1962, 1963) that the lymphoid tissues of tolerant mice, when examined by chromosome analysis, were almost wholly replaced by donor cells may well have reflected the death of many of the host cells as a result of a graft-versus-host reaction. In distinction from the surprisingly high percentage of donor lymphoid cells in their tolerant animals, these investigators

could not find any donor cells in mice that had completely rejected skin grafts which were formerly tolerated.

In a series of experiments on the quantitative analysis of the chimaeric state in mice, Nakić and his collaborators (Nakić, Mikuska, Kastelan, Springer and Silobrcić, 1970) have shown by means of chromosomal analysis that mice injected with F_1 hybrid cells had uniformly low frequencies of donor cells in the spleen, thymus, bone marrow and caecal lymphoid tissue. Relatively higher proportions of donor cells were identified in the lymph node and Peyer's patches during the first three weeks of life, but there was a subsequent steady decline in donor cell content. Despite this marked fall in the degree of chimaerism, specific tolerance, as tested by repeated skin grafting, was fully maintained.

Cellular chimaerism has also been demonstrated in adult mice in which homograft tolerance had been induced by sublethal irradiation and the injection of donor-type spleen cells in high dosage (Michie and Woodruff, 1962), and in adult mice rendered homograft tolerant by use of urethan and sublethal irradiation followed by injection of spleen cells (Davis and Cole, 1963).

2) The occurrence of chimaerism in the absence of tolerance

Although the experiments reviewed above imply that the tolerant state is associated with persistent cellular chimaerism, dissociation between cellular chimaerism and skin graft tolerance has been described. This phenomenon of split-tolerance was first observed in A strain mice which had been

injected at birth with (CBA x C57) F_1 spleen cells (Billingham and Brent, 1959; Brent and Courtenay, 1962). Such animals were found to remain tolerant of CBA skin grafts while only weakly tolerant of C57 or (CBA x C57) F_1 grafts. Examination of their chimaeric status indicated that, even after such animals had rejected both F_1 hybrid and C57 strain skin homografts, they remained chimaeric. That is, cells of F_1 hybrid type had survived in the spleen although the host had clearly succeeded in rejecting both F_1 hybrid and C57 skin grafts. It was noted that the genetic relationship between donor and recipient was of importance in tolerance induction and that C57 antigens were more "foreign" to A strain recipients than were CBA antigens. They concluded that F_1 hybrid type cells in the spleen were inherently less susceptible to immune attack than were skin grafts (Brent and Courtenay, 1962).

Further evidence that dissociation of skin homograft tolerance and cellular chimaerism may occur was provided by BN rats which had been neonatally inoculated with lymph node or spleen cells of (Lewis x BN) F_1 hybrid origin (Billingham, Defendi, Silvers and Steinmuller, 1962). It was found that some feebly tolerant animals still appeared to have foreign leucocytes in their blood after they had rejected skin homografts. Furthermore, a high proportion of these rats succumbed to a graft-versus-host reaction when specifically sensitized lymphocytes from Lewis donors were administered to them. These results were interpreted as indicating that the rats were not tolerant of skin homografts, but were tolerant

of the mesenchymal cells inoculated at birth.

Recently, Silvers and his collaborators demonstrated that BN rats which had been inoculated at birth with (Lewis x BN) F_1 hybrid strain lymph node cells retained a normal capacity to reject Lewis skin grafts even though their peripheral blood lymphocytes were significantly less reactive to Lewis cells in mixed lymphocyte reactions (Silvers, Lubaroff, Wilson and Fox, 1970). Situations where cellular chimaerism persisted in the absence of tolerance of skin homografts have also been described in mice (Warner, Herzenberg, Cole and Davis, 1965), cattle (Stone, Cragle, Swanson and Brown, 1965; Stone, Cragle, Johnson, Bacon, Bendel and Korda, 1971) and man (Beilby, Cade, Jelliffe, Parkin and Stewart, 1960).

These studies on the dissociation of cellular chimaerism and skin homograft tolerance have been interpreted to indicate that persistent chimaerism with respect to a cellular inoculum prepared from one organ is compatible with weak or even imperceptible tolerance of a subsequent graft of a different tissue, if the former cells fail to express adequately all the antigens effectively expressed by the latter (Billingham, Defendi, Silvers and Steinmuller, 1962). Dresser and Mitchison also indicated that split tolerance might be the result of heterogeneity of response to a mixture of antigens of heterogeneous strength (Dresser and Mitchison, 1968).

3) The occurrence of tolerance in the absence of chimaerism

In a series of experiments on the male specific transplantation antigen in mice, Billingham and his

collaborators have shown that, although homograft tolerance of this antigen can be induced in female C57BL strain mice with bone marrow cells from homologous male mice, cellular chimaerism is infrequent in these females. They tended to reject skin homografts from mice of the bone marrow donor strain with normal vigour but were tolerant of syngeneic male skin grafts (Billingham and Silvers, 1960). It was reasoned that, if such animals were indeed non-chimaeric, the placing of normal females in parabiotic union with them should neither sensitize nor render the normal parabionts tolerant of the male specific antigen. Paradoxically, although chimaerism could not be detected in the tissues of the tolerant partners, some 40% of their normal female partners were rendered tolerant, shorter periods of union appearing to be more effective than longer ones (Billingham, Silvers and Wilson, 1965).

Nisbet has recently obtained similar evidence (Nisbet, 1967, 1971). It was found that homograft tolerance which had been induced by means of parabiosis between parental strain and F_1 hybrid strain mice might not be associated with cellular chimaerism, as determined by chromosomal analysis. Furthermore, the tolerant state induced in this manner could be readily transferred to syngeneic neonatal mice provided that the genetic disparity was weak (Nisbet, 1971).

The experiments described above are the only reports of the transfer of the tolerant state from tolerant to normal animals in the absence of detectable cellular chimaerism. Despite the failure to demonstrate donor cells in these experiments, it is not possible to exclude cellular chimaerism

unequivocally. There are other experimental situations in which chimaerism can definitely be excluded. The repeated injections of non-viable spleen material into either adult or neonatal mice resulted in tolerance of allogeneic tissue grafts (Martinez, Smith, Blaese and Good, 1963). An attempt was made to ascertain whether or not this state of non-reactivity could be transferred to neonatal syngeneic mice by injecting the latter animals with spleen cells from mice previously made tolerant by injection of disrupted spleen cells (Martinez, Smith, Blaese and Good, 1963). Homograft tolerance induced in mice by the intravenous injection at birth of viable lymphoreticular cells, and also tolerance induced by parabiosis, can be transferred to neonatal syngeneic animals by injecting spleen cells from the tolerant donors (Martinez, Smith, Aust, Mariani and Good, 1958; Martinez, Shapiro and Good, 1961). This phenomenon has been attributed to the transfer of viable lymphoreticular cells replicating in the tolerant recipient and reflecting the chimaeric state. It was found that spleen cells from mice made tolerant, either as adults or as neonates, by the injection of disrupted spleen cell preparations were incapable of transferring the tolerant state to syngeneic newborn recipients. These observations suggest that the antigens responsible for the tolerant state are only very slowly metabolized, or, alternatively, that a skin graft is itself able to maintain the tolerant state once tolerance has been established by injection of the disrupted cell preparation.

D. The immunological reactivity of the lymphoid cells of tolerant animals

As a means of defining more closely the basis of homograft tolerance, parameters other than the acceptance of skin grafts have been used to assess the immune status of tolerant animals.

1) The graft-versus-host reaction

The immunological reactivity of the lymphoid cells of tolerant animals has been examined by means of graft-versus-host assays. Michie, Woodruff and Zeiss (1961) were the first to demonstrate that spleen cells from adult mice were incapable of evoking splenomegaly in new born mice belonging to a strain of which the spleen cell donor was tolerant.

Subsequently, it has been shown that small lymphocytes from immunologically tolerant animals of parental strain fail to cause any histological or clinical signs of a graft-versus-host reaction when they are injected intravenously into rats of the tolerated strain (Gowans, McGregor and Cowen, 1963; McCullagh and Gowans, 1966). The transformation normally observed in small lymphocytes of parental strain after their transfer to F_1 hybrid strain recipients does not occur if the donor is immunologically tolerant of F_1 hybrid strain tissues (Elkins, 1964; Gowans, 1965).

That the graft-versus-host performance of an animal's lymphocytes can reflect quantitatively and specifically a state of tolerance toward histocompatibility antigens has been confirmed recently by Bildsøe, Ford, Pettirossi and Simonsen (1971) and Atkins and Ford (1972) using the popliteal lymph

node weight assay described by Ford, Burr and Simonsen (1970).

2) Mixed lymphocyte culture

If peripheral blood lymphocytes from two unrelated animals are mixed and cultured for several days, a significant proportion of the mixed cell population undergoes transformation to blastoid forms, incorporates tritiated thymidine and enters mitosis. This proliferative response has been attributed to the reaction of antigen-sensitive cells to foreign histocompatibility antigens. Since lymphocytes from tolerant donors fail to transform into pyroninophilic cells or to induce runt disease, after transfer to F_1 hybrid strain hosts, it has been suggested that cell transformation on the part of small lymphocytes is an essential part of the initial phase of the primary immunological response to transplantation antigens. Examination of the activity of cells from tolerant donors in the mixed lymphocyte reaction has shown that lymphocytes from such animals are specifically unreactive against cells bearing antigens of the tolerance-inducing strain while retaining normal reactivity towards third-party homologous lymphocytes (Wilson, Silvers and Nowell, 1967; Schwarz, 1968; Wilson and Nowell, 1970). These results are in accord with the conclusion that the immunological deficiency of a tolerant animal resides in its lymphocyte population.

3) Cytotoxic effects in vitro of lymphocytes from tolerant donors

Holm and Perlmann (1965) and Möller (1965) found that unsensitized spleen or lymph node cells could rapidly destroy appropriate target cells provided aggregation between

lymphocytes and target cells had been brought about by the addition to the cultures of phytohaemagglutinin. Destruction did not occur in syngeneic combinations, but F_1 hybrid strain lymphoid cells attacked parental-strain targets, suggesting that the phenomenon depended on structural differences between the contestants rather than upon a "conventional" immune response (Möller, 1965). Möller and Lapp studied the cytotoxic capacity of lymphoid cells from specifically tolerant mice in tissue culture in the presence of phytohaemagglutinin (Möller and Lapp, 1969). It was found that lymphoid cells from tolerant A strain mice carrying (A x CBA) F_1 hybrid strain skin grafts were competent to cause destruction of both (A x CBA) F_1 hybrid strain and CBA strain fibroblast target cells in tissue culture in the presence of phytohaemagglutinin. More recent observations on the cytotoxic capacity of lymphoid cells from tolerant donors will be discussed in Section 1G.

The interpretation of the results obtained with these different means of assessing the reactivity of cells from tolerant animals is confused. A barrier composed of several weak histocompatibility loci may mimic a strong locus barrier in response to skin grafting although not in graft-versus-host or mixed lymphocyte reactions (Sillers and Billingham, 1969a; Simonsen, 1970). Moreover, it should be mentioned that after induction of tolerance by neonatal injection of F_1 hybrid strain cells, the absence of stimulation in the mixed lymphocyte reaction does not necessarily indicate that tolerance to a skin graft is present (Sillers, Lubaroff, Wilson and Fox, 1970).

It is clear from other types of experiments that graft-versus-host and mixed lymphocyte assays do not measure identical phenomena. For example, when spleen cells were examined four days after intraperitoneal immunization with allogeneic spleen cells (at the height of the large pyroninophilic cell response), a large increase in stimulation was found in mixed lymphocyte culture (Virolainen, Häyry and Defendi, 1969) but in a very similar experiment no increment was found in graft-versus-host assays (Ford and Simonsen, 1971).

4) The susceptibility of tolerant, adult animals to graft-versus-host disease following the transfer of tolerated, allogeneic lymphocytes

Tolerant animals are highly susceptible to runt disease following their inoculation with lymphoid cells from normal or specifically sensitized mice of the tolerated strain. Billingham and Silvers (1961b) injected $50-100 \times 10^6$ lymph node cells from CBA-sensitized A strain mice into CBA mice tolerant of (CBA x A) F_1 tissues. All of these injected tolerant CBA mice succumbed to a condition resembling graft-versus-host disease within 2-3 weeks of this cell transfer. Of ten mice which received grafts of both CBA and A strain skin at the time of lymph node cell injection, six rejected their syngeneic (CBA) grafts. Nevertheless, all A strain grafts remained in perfect condition for the duration of their hosts' life. This observation of the acceptance of homografts concurrent with the rejection of syngeneic grafts was fully confirmed by Stastny, Stenbridge and Ziff (1963)

in the rat. Careful studies by Martinez, Smith and Good (1961) suggested that this disease in tolerant animals was similar in nature to the graft-versus-host reaction observed in three other types of host, namely:

1. newborn animals injected with homologous lymphoid cells;
2. F_1 hybrid strain mice receiving inocula of lymphoid cells from mature donors of either parental strain; and
3. mice rescued from lethal irradiation by the inoculation of homologous bone marrow cells.

E. Termination of homograft tolerance

Termination of the state of homograft tolerance, with rejection of skin grafts that were previously accepted, has been achieved in a number of ways. The requirements for such termination and its sequels give some indications as to the nature of the tolerant state.

1) Termination of the tolerant state by transfer of cells from normal syngeneic donors

Billingham, Brent and Medawar (1956a) first achieved the termination of homograft tolerance, as evidenced by destruction of tolerated skin homografts, by transfer of either sensitized, or normal, lymph node cells from syngeneic mice. They found that abrogation of tolerance could be more quickly accomplished if the lymph node cells were obtained from specifically sensitized donors, and that specifically sensitized lymph node cells from donors of a strain different to that of the tolerant mouse could not abrogate the tolerant state. It was also noted that second grafts placed on

"terminated tolerant" mice were very rapidly rejected.

These important observations led Medawar and his colleagues to suggest that tolerance is due to a central failure of the mechanism of immunological response, and not to some interference with the homograft reaction at a "peripheral" level.

a) The type of cell which initiates the destruction of skin homografts in tolerant animals. The initial study of abrogation of homograft tolerance by means of normal, syngeneic lymph node cells suggested that the deficiency of the tolerant animal lies in its lymphoid tissue. Subsequent work has shown that the lymphoid tissue of tolerant mice can be re-equipped equally well by injections of peripheral blood leucocytes from normal syngeneic donors (Billingham, Silvers and Wilson, 1962; Billingham, Silvers and Wilson, 1963). This suggests that the cell-type which can initiate the destruction of skin homografts in tolerant animals is a lymphocyte, since lymphocytes are the cells which lymphoid tissue normally contributes to the blood. Billingham, Silvers and Wilson (1962, 1963) and Gowans and his collaborators (Gowans, Gesner and McGregor, 1961; Gowans, McGregor, Cowen and Ford, 1962; Gowans, McGregor and Cowen, 1963) have formally confirmed this point by demonstrating the effectiveness of cells from the thoracic duct of normal syngeneic donors in abrogating tolerance of skin homografts in rats. The use of inocula in which the proportion of large lymphocytes in thoracic duct lymph had been greatly reduced provided strong evidence that the effective cell type was the small

lymphocyte. Thus, the intravenous injection of 50×10^6 small lymphocytes containing about 0.1% of medium lymphocytes led to the destruction of long-standing skin homografts in nine out of twelve tolerant rats (Gowans and McGregor, 1965).

b) Relative efficiency of lymphoid cells from normal and sensitized donors. If similar numbers of lymphoid cells from normal and sensitized donors are transferred, those from the latter are more effective as evidenced by the shorter time required for the abrogation of the tolerant state. To explain this observation, Billingham, Brent and Medawar (1956a) suggested that, whereas the state of immunity is pre-existing, and has merely to be expressed, normal cells must be actively sensitized by antigenic matter issuing from tolerated skin homografts and chimaeric cells before they can exert any effect on tolerated tissues. Similarly, Gowans suggested that, when "sensitized" lymphocytes are injected, a tolerant animal is populated with a supply of cells which are already capable of destroying grafts (1961).

c) Variability in graft rejection following transfer of normal lymphocytes. Studies on abrogation of homograft tolerance have shown that tolerance is not always abrogated and that the survival times of the grafts which are destroyed show a considerable spread. For example, in one investigation, an injection of 10^9 small lymphocytes destroyed four grafts with approximately the tempo of a normal first-set response (8-11 days), but one graft was unaffected (survival > 100 days). 4×10^8 small lymphocytes destroyed one graft in 12 days and another in 44 days (Gowans, McGregor and Cowen,

1963). Billingham, Silvers and Wilson (1963) also found that, whereas 15×10^6 normal lymph node cells destroyed 3 out of 5 tolerated skin grafts, 120×10^6 of the same type of cells destroyed only 1 out of 3 skin homografts. Furthermore, Silvers (1970) injected one donor equivalent of normal lymphoid cells prepared from axillary, inguinal, cervical and mesenteric nodes and spleen from a single donor into tolerant mice and showed that 3 out of 10 tolerated skin homografts survived throughout the 200 day observation period while the survival times of 7 rejected skin homografts ranged from 20 to 136 days.

It has been suggested that this variability of response may be a reflection of the chimaeric status of the tolerant host (Billingham, Silvers and Wilson, 1963; Gowans, McGregor and Cowen, 1963). It seems likely that the donor lymphocytes would be confronted in the tolerant recipient, not only by the foreign skin grafts, but also by foreign cells which were derived from the tolerance-inducing neonatal inoculum. Consequently, the amount of foreign antigen encountered by a standard inoculum of lymphocytes might vary from animal to animal. When the amount is large the immunological attack might exhaust itself without attacking the skin graft (Gowans and McGregor, 1965). On the other hand, Billingham (1963) has pointed out that the presence of some chimaeric tissue may considerably aid the adoptive destruction of the tolerated graft. The results of their studies on abrogation of tolerance of the Y-factor lend considerable weight to this interpretation (Billingham, Silvers and Wilson, 1965). They found that, when tolerant hosts were demonstrable chimaeras,

tolerance of male specific antigen could be abrogated by adoptive immunization, whereas putatively non-chimaeric, or low level chimaeric tolerant females were refractory to the transfer of three donor-equivalents of specifically immune lymphoid cells. However non-chimaeric, or low level chimaeric females could be rendered susceptible to termination of tolerance with syngeneic cells. This could be achieved either by transplanting a second isograft of male skin at the time of cell transfer, or by injecting them with appropriate numbers of syngeneic male spleen cells prior to, or concomitant with, the transfer of the immune lymph node cells.

These observations were interpreted as indicating that the transferred lymphoid cell population did not include enough cells capable of immediately circulating in the blood stream and infiltrating and attacking a well-established target graft. Some supporting evidence has come from recent experiments using an Ag-B compatible combination of Lewis and Fisher rats (Silvers and Billingham, 1969a). It was found that animals rendered tolerant by the injection of very low dosages of cells were more resistant to termination of the tolerant state than animals whose tolerance had been induced by larger numbers of donor cells. Whereas the transfer of 10^8 normal lymphoid cells led to the destruction of long-standing skin homografts in 2 out of 3 rats in which tolerance was induced with 2×10^7 spleen cells, similar numbers of lymphoid cells were totally ineffective in abrogating tolerance in 3 rats in which tolerance had been

induced with only 0.25×10^6 spleen cells.

d) Histological features of the hosts' lymphoid tissues during the termination of tolerance following the transfer of thoracic duct lymphocytes from normal donors.

Gowans, McGregor and Cowen (1963) studied the cellular changes which accompanied the adoptive destruction of homografts in immunologically tolerant rats. A standard dose of 5×10^8 syngeneic thoracic duct cells from normal donors was injected intravenously into a number of tolerant parental-strain rats bearing long-standing homografts of F_1 hybrid strain skin.

The first change in hitherto tolerated F_1 hybrid strain grafts occurred about 4 days after the injection and consisted of a perivascular accumulation of mononuclear cells in the base of the grafts. By the fifth and sixth days mononuclear cells had infiltrated throughout the dermis. When histological evidence of necrosis was seen, the grafts were considerably less cellular than homografts on normal rats in which destructive changes had advanced to the same degree.

The cellular changes in lymphoid tissue were not restricted to the regional node, and transformation of some of the injected small lymphocytes into large pyroninophilic cells was seen within 24 hours in the splenic white pulp and in several non-regional nodes as well as in the regional node. These cellular changes in the lymphoid tissues appeared to be quite similar to the graft-versus-host reaction which follows the injection of parental-strain small lymphocytes into F_1

hybrid strain rats (Gowans, 1962). Gowans and his colleagues concluded that the injected lymphocytes were reacting against the chimaeric cells in the lymphoid tissue of the host and that the effector mechanism responsible for destroying the graft was generated by this "graft-versus-host" reaction.

e) The appearance of immunologically competent host cells following termination of tolerance. The experiments described so far did not give any indication whether specifically reactive cells of host origin could appear in association with rejection of the tolerated skin homografts. This point has been examined recently (Silvers and Billingham, 1970). Lewis rats were rendered tolerant of BN and DA strain skin homografts by the neonatal injection of (BN x DA) F_1 hybrid strain bone marrow cells. When these doubly tolerant animals were inoculated with lymphoid cells from either (Lewis x DA) F_1 hybrid strain animals sensitized against BN strain tissues, or (Lewis x BN) F_1 hybrid strain rats sensitized against DA strain tissues, both types of long-established grafts were rejected. Silvers and Billingham inferred that both types of specifically sensitized F_1 hybrid strain cells, when transferred to Lewis rats tolerant of (BN x DA) F_1 hybrid strain tissues attacked and eliminated the descendants of the (BN x DA) F_1 hybrid strain cells. It was concluded that, as a consequence of elimination of these chimaeric cells, immunologically competent host cells ultimately appeared. The reason proposed for the failure of the inoculated (Lewis x DA) F_1 hybrid or (Lewis x BN) F_1 hybrid strain cells to deputize for the presumably eliminated (BN x DA) F_1 hybrid

strain bone marrow cells and maintain the chimaeric status required for tolerance was the relative ineffectiveness of lymph node, and spleen cells, as compared with bone marrow cells, in inducing tolerance of skin grafts in Ag-B incompatible situations.

2) The termination of the tolerant state by means of parabiosis with a normal partner

Parabiosis of tolerant mice and rats with normal or specifically sensitized syngeneic animals was an effective means of destroying established grafts in tolerant animals (Billingham, Silvers and Wilson, 1963). This result was considered to be comparable to that observed following the transfer of normal, syngeneic lymphoid cells.

In the preceding experiments, non-tolerant animals were united to tolerant animals in which the tolerant state had been induced with a cell inoculum from a donor differing with respect to strong histocompatibility factors. In contrast to this situation, parabiosis of normal females with females rendered tolerant by the neonatal inoculation of isologous bone marrow cells from male donors resulted in the acquisition of tolerance of male specific antigens by some 80% of the normal females (Billingham, Silvers and Wilson, 1965). Passage of tolerance of non H-2 antigens by parabiosis has also been reported from studies on C3H and Ce mice (Martinez, Smith, Shapiro and Good, 1959).

The transfer of tolerance as a result of parabiosis has been attributed to the transfer of viable chimaeric donor cells replicating in the tolerant recipient. This conclusion was supported by the observation that no normal female mice

which had been parabiosed with females rendered tolerant of male specific antigen through multiparity became tolerant (Billingham, Silvers and Wilson, 1965). Furthermore, specific immunologic tolerance induced with intact cells in neonatal animals, or in adult life, is transferrable to newborn syngeneic recipients, while that induced during adult life with disrupted spleen cells cannot be transferred in this way (Martinez, Smith, Blaese and Good, 1963).

3) Termination of the tolerant state by the transfer of isoantisera

Brent and Medawar (1962) injected isoantiserum directed against graft antigens into tolerant mice and found that none of the tolerated skin homografts were rejected but 2 out of the 23 tolerant hosts lost their chimaerism. They predicted the possibility of elimination of tolerated chimaeric cells by isoantiserum. Subsequently, Lewis anti-DA antiserum was injected into 16 Lewis rats tolerant of DA tissues (Feldman, Pick, Lee, Silvers and Wilson, 1968). Once again, all of the tolerant animals failed to reject their tolerated skin homografts. It was found, however, that the intravenous injection of isoantiserum was followed within 12 hours by transitory macroscopic changes in the long-surviving skin homografts on 6 of the 16 recipients. Graft pathology was histologically typical of early and mild homograft reactions, there being focal necrosis of the epidermis and hair shaft epithelium, vascular dilatation in the base of the graft, and infiltration with mononuclear cells. These changes were sharply limited to the tolerated graft.

Repeated infusions of this antiserum over a period of 4 days failed to augment these changes and recovery was observed even while serum administration continued. These two experiments implied that both chimaeric cells and skin homografts could be injured to some extent by relatively small amounts of isoantiserum. Recently, Hasek and his colleagues (Hasek, Skamene, Karakoz, Chutná, Nouza, Bubeník, Sovová, Nemec and Jonák, 1968) injected large amounts of specific isoantiserum (equivalent to as much as 20% of the tolerant recipient's body weight) and succeeded in achieving rejection of tolerated skin homografts in 91 out of 117 ducks. Transfer of isoantibodies produced oedema and haemorrhage in the graft within 2-3 hours. In the early phase after injection of serum the histological picture of the tolerated grafts undergoing rejection had all of the characteristics of an Arthus reaction accompanied by polymorphonuclear infiltration. In the later phases, lymphocytes were present both within and outside the vessels of the sloughing graft. Second grafts transplanted 2, 4 and 6 weeks after the first grafts had been rejected, were not accepted.

These observations implied, by the rapidity of the process, that rejection of tolerated cells after the injection of antibody was caused by the direct effect of antibody. It was also inferred that the rejection of tolerant homografts was not due to the action of serum cytotoxic antibodies, since absorption of serum with syngeneic spleen cells removed its destructive effects on the graft, but did not reduce the titre of cytotoxic antibodies.

Lubaroff and Silvers (1970) undertook a similar

study by injecting relatively large amounts of isoantiserum into tolerant rats. A total of about 6.0 ml of isoantiserum was injected intraperitoneally over a 7 day period. Within 12 hr after transfer of specific isoantiserum to 22 rats bearing Ag-B incompatible grafts, the grafts became inflamed and some developed minor lesions. However, only two instances of acute rejection were observed. Subsequently, the grafts underwent total destruction in 10 of these 22 animals, this process being deferred for as long as 49 days after the serum was transferred. Rats whose grafts still survived 50 days after serum transfer were retreated with specific isoantiserum. This increased the number of rejections to 17 of the 22.

An additional interesting finding was that Lewis anti-DA serum led to the destruction of long-standing skin homografts of both DA and BN strains in all 4 Lewis rats in which tolerance had been induced by the neonatal inoculation of (DA x BN) F_1 hybrid strain bone marrow cells. In contrast with these results, rats tolerant of Ag-B compatible homografts were completely refractory to attempts to terminate the tolerant state.

Animals in which tolerance had been terminated were rechallenged 50 to 100 days later with skin from the original donor strain. These grafts were promptly and consistently rejected. Lubaroff and Silvers considered possible explanations for the significant delay in graft destruction mediated by isoantiserum as compared with the prompt destruction of grafts by the transfer of sensitized cells and suggested that the tolerated grafts had been destroyed by immunologically

competent host cells which had developed after the elimination of chimaerism. It must be noted that, regardless of the mechanisms underlying the results reported by Hasek et al. (1968) and Lubaroff and Silvers (1970), such animals in which tolerance had been abrogated by the isoantiserum did not regain tolerance to second grafts. As these rats had not at any time received lymphocytes, it is evident that any immunological activity must be attributable to host cells.

4) Termination of the tolerant state by means of irradiation

Fefer and Nossal (1962) were the first to demonstrate that X-irradiation could abrogate homograft tolerance in mice. They induced tolerance by perinatal exposure of animals. At 39 days of age, the treated mice received a total of 350 to 450 r X-irradiation and, 17 days after irradiation, they were transplanted with a single skin homograft from the tolerance-inducing donor strain. It was found that, whereas X-irradiation prolonged the graft survival time in non-tolerant control animals, X-irradiated partially tolerant mice rejected their skin homografts as rapidly as did unirradiated control mice. Skin homografts on completely tolerant animals, however, appeared to be unaffected by X-irradiation.

It was suggested that the neonatal injection of homologous cells may have induced tolerance in the majority of the recipient's potentially immunologically competent cells, while leaving a small number unmodified. The release of homologous antigens from persisting donor cells might have immunized this small residual population, but, if the number of sensitized cells was inadequate to effect a vigorous

rejection of a skin homograft, partial tolerance resulted. These immunized cells may have been more radioresistant than the bulk of the lymphoid cell population (Taliaferro and Taliaferro, 1951), giving them a marked selective advantage during post-irradiation proliferation, while the release of homologous antigens from irradiation-damaged donor-type cells could have provided a further proliferative stimulus. This model predicts that completely tolerant mice which lacked any residual unmodified host cells should not be abrogated by irradiation.

Using tolerant rats which had been bearing skin homografts for more than 50 days, the efficacy of X-irradiation in abrogating tolerance has been examined (Feldman, Pick, Lee, Silvers and Wilson, 1968). Two regimes, namely irradiation alone and the combination of irradiation plus the transfer of normal or sensitized syngeneic cells, were employed. X-irradiation of 550 r alone was followed by skin homograft rejection in 6 out of 16 rats. Irradiation was shown to facilitate the destruction of tolerated grafts by transferred syngeneic cells. Using this combined treatment, rejection of homografts was accomplished more readily with fewer cells and at an accelerated tempo compared with either irradiation or cell transfer alone.

Silvers (1970) attempted the serial passage of homograft immunity in tolerant mice, using irradiation of prospective tolerant recipients. He found that the prior exposure of tolerant recipients to a relatively low dose of X-irradiation (300 r) markedly augmented the capacity of both

normal and sensitized syngeneic lymphoid cells to abrogate tolerance of skin homografts when passaged through successive tolerant hosts. In contrast, none of the 16 tolerant mice which received irradiation alone, rejected its graft. This effect of irradiation was attributed to the creation of "Lebensraum" for the transferred cells enabling them to become established and proliferate. It was also suggested that irradiating the tolerant animal reduced its level of chimaerism so that a greater number of the inoculated, immunologically competent cells were available to react directly with the foreign skin graft and destroyed it.

F. The immunological competence of the lymphoid cells of animals in which the tolerant state has been terminated

Despite the assistance which such information might provide for understanding the cellular nature of tolerance, there has been very little investigation of the immunological reactivity of cells from tolerant animals after the termination of this state. It is known that second grafts are rejected by such animals. As an extension of this observation Silvers (1970) studied the capacity of lymphoid cells from tolerant mice in which the tolerant state had been abrogated as a result of the transfer of normal or specifically sensitized syngeneic cells to terminate the tolerant state when they were themselves transferred to other tolerant mice. It was found that lymphoid cells from "terminated-tolerant" mice failed to effect the rejection of tolerated skin homografts beyond the second "generation" of recipients unless those tolerant recipients had been irradiated before receiving cells from

"terminated-tolerant" mice. This inability to pass the capacity to reject homografts is reminiscent of the difficulty encountered when the serial transfer of graft-versus-host activity has been attempted in various experimental systems (Burnet and Boyer, 1960; Dineen, 1961; Papermaster, Bradley, Watson and Good, 1962; Billingham, Defendi, Silvers and Steinmuller, 1962; Streilein and Billingham, 1970).

Using the injection of parental strain lymphoid cells under the kidney capsule of F_1 hybrid strain rats as an assay system, Elkins (1972) has reported that the graft-versus-host reactivity of lymphoid cells from rats which were originally tolerant remains at very low levels for at least four months after graft rejection. Some evidence was also obtained to suggest that lymphoid cells obtained from "terminated-tolerant" animals could exert a suppressive effect on the part of lymphocytes from untreated tolerant rats, it was inferred that the cells exerting this suppressive influence had been generated as a result of the termination of tolerance and the elimination of chimaeric cells. As a result of this observation, Elkins suggested that a suppressive effect could be responsible for the difficulty experienced by Silvers in passaging homograft immunity through a series of tolerant animals. In view of the failure to demonstrate suppressor activity on the part of lymphocytes from tolerant chimaeras, it was inferred that the manifestation of specific tolerance by these cells in graft-versus-host and mixed lymphocyte reactions reflects the absence of specifically reactive cells rather than inhibition of the latter by

blocking factors in the serum.

G. The mechanism of homograft tolerance

Two extreme possibilities have been proposed to explain the specific absence of rejection of otherwise susceptible skin homografts and chimaeric donor cells in homograft tolerant animals. One possibility is that tolerance to tissue homografts is attributable to an absence of any type of immunological response, as a result of the elimination of specifically reactive clones of cells. An alternative is that the state of tolerance represents an active response manifested by the formation of suppressive humoral factors, or by the generation of suppressor cells, that interfere with the cells involved in the homograft reaction.

Evidence in favour of the existence of suppressive humoral factors was presented by Voisin, Kinsky and Maillard (1968) who demonstrated that the lymphoid tissues of animals tolerant of allogeneic cells were in a state of reactivity more closely resembling that of immune than normal animals. They arrived at this conclusion, by studying the occurrence of pyroninophilic cells and of immunoglobulin producing cells. They also demonstrated that sera from tolerant animals could specifically promote the establishment and growth of donor strain sarcomata grafted to normal mice syngeneic with the serum donor. However, sera from tolerant mice failed to evince reactivity towards tissues from the tolerated strain when tested for haemagglutination, haemolysis, cytotoxicity, or passive cutaneous anaphylaxis.

More recently, Hellström, Hellström and Allison

(1971) have reported that lymphoid cells from CBA mice tolerant of A strain tissues exhibit some in vitro cytotoxicity for A strain target cells, and that this cytotoxicity is specifically inhibited by serum from the tolerant animals. These experiments implied that the lymphoid cells of the tolerant mice were sensitized against A strain tissues as lymphoid cells from normal (unsensitized) mice were not cytotoxic in the in vitro assay system used. The experiments of Voisin et al. and of Hellström et al. support the contention that procedures which induce tolerance do so by invoking active immunological reactions. These reactions may entail the production of specifically immune cells and of serum factors capable of suppressing the homograft reaction mediated by immune cells.

The possibility that tolerant animals may produce serum factors capable of prolonging homograft survival brings into question the relationship of tolerance to immunological enhancement. Enhancement has been defined as the successful establishment, or delayed rejection, of an allograft as a consequence of the presence of specific antiserum in the host (Kaliss, 1962). The basic conditions for inducing enhancement have been extensively studied in inbred mice and have been reviewed in detail (Kaliss, 1958). Progressive growth of tumor grafts in certain strains in which they normally would be rejected may follow active immunization of the host with lyophilized tumor tissue or normal tissue of the strain to which the tumor is indigenous. Enhancement may also follow passive immunization of the host with isoantiserum directed

against the tumor grafts. Although enhancement can also apply in the case of normal tissues it is often less easy to demonstrate (Brent and Medawar, 1962; Möller, 1964). However, several investigators have succeeded in prolonging the survival of normal tissue grafts by antigen pretreatment (Billingham, Brent and Medawar, 1956b; Nelson, 1962; Heslop, 1966) and by specific antiserum (Brent and Medawar, 1962).

More recently, indefinite survival of renal allografts in rats has been produced by the administration of alloantibody directed against donor tissue antigens (Stuart, Saitoh and Fitch, 1968; French and Batchelor, 1969; Lucas, Markley and Travis, 1970) and, by pretreatment with donor tissue antigens (Zimmerman, 1971; Wilson, Maggs, Vanwijck, Shaipanich, Holl-Allen, Lukl and Simonian, 1971; Marquet, Heystek and Tinbergen, 1971; Fabre and Morris, 1972). Enhancement has also been proposed to explain observations such as the protective effect conferred by isoantiserum directed against host tissue against the otherwise lethal graft-versus-host disease of F_1 hybrid animals induced by parental strain immunologically competent cells (Voisin and Kinsky, 1962; Batchelor and Howard, 1965).

As previously described, lymphoid cells from tolerant animals lack graft-versus-host reactivity towards otherwise susceptible recipients of the tolerated strain. The graft-versus-host activity of lymphoid cells from rats bearing histoincompatible kidney grafts in which the unresponsive state seems to be due to enhancement has been examined quite recently by two groups. French, Batchelor

and Watt (1971), using the assay systems of both Elkins (1964) and Ford et al. (1970), demonstrated that lymphoid cells from rats bearing an isoantibody-enhanced kidney were able to mount graft-versus-host reactions quantitatively similar to those caused by cells from control rats, and suggested that a central failure of the immune mechanism was not involved in the prolonged survival of these grafts. One finding of particular interest was that, although immunization of rats bearing enhanced kidneys with spleen cells from a rat of the same strain as the kidney donor was followed by a rise in cytotoxic antibody titer, it had no effect on the survival of the enhanced kidney as estimated by blood urea levels.

Bildsøe, Ford, Pettirossi and Simonsen (1971) undertook a similar study using AS2 rats bearing AgB incompatible AS kidneys which are known to survive for many months without immunosuppressive treatment (Salaman, Elves and Festenstein, 1971). The graft-versus-host activity of spleen cells from these rats was measured by the popliteal lymph node assay of Ford et al. (1970). The graft-versus-host activity of the lymphoid cells of rats rendered tolerant by classical procedures was also examined. Whereas the kidney recipients showed normal or slightly reduced reactivity, lymphoid cells of rats in which tolerance had been induced by neonatal injections were almost completely lacking in reactivity. Both of these groups reached a similar conclusion, namely that the long-term survival of organ grafts in the face of a vigorous cellular response against the donor's antigens may have been attributable to enhancing antibodies.

The possibility that potentially reactive donor lymphocytes exist in tolerant animals but remain inactive when their reactivity is tested by transfer to F_1 hybrids because of the presence of inhibitory antibody secreted by other donor cells has been considered (Atkins and Ford, 1972). If such a mechanism was operative, the graft-versus-host activity of lymphocytes from a normal rat, when intimately mixed with an excess of tolerant lymphocytes, should also be impaired by inhibitory antibody. Attempts to demonstrate such an impairment were negative, including experiments in which normal cells to be injected were suspended in tolerant serum or exposed to a 10~12 fold excess of tolerant cells.

Elkins (1972) undertook a similar study on lymphoid cells of tolerant rats in which the tolerant state had been abrogated by means of the transfer of normal, syngeneic cells. It was found that, if normally reactive cells were mixed with an excess of lymphoid cells from "terminated-tolerant" rats and injected under the kidney capsule, the capacity of the former to mount a graft-versus-host response could be completely suppressed. However, as with the results obtained by Atkins and Ford (1972), lymphoid cells from untreated tolerant rats failed to influence the reactivity of normal lymphoid cells. Consequently, it seems unlikely that suppressor cells appearing after the termination of tolerance by the transfer of normal syngeneic cells play a major role in maintaining the unresponsive state in uninjected tolerant animals (Elkins, 1972). Extensive studies to demonstrate an in vivo role for serum blocking factors in tolerant mice were

performed by Brent, Brooks, Lubling and Thomas (1972) with negative results. Of particular interest was the failure to modify the behaviour of relatively small numbers of normal lymphoid cells in a graft-versus-host assay based on splenomegaly in neonates, and the inability to facilitate tolerance induction in neonatal mice. In both situations the amounts of tolerant serum injected represented a high proportion of the host's own plasma volume.

To explain this apparent contradiction between in vivo and in vitro data, Brent et al. (1972) have postulated the existence of a subpopulation of antigen-sensitive cells that is resistant to tolerance induction but remains susceptible to the action of blocking factors. If this subpopulation represents a very small fraction of the total number of antigen-sensitive cells, the majority having become tolerant, it might not be adequate to be detected in the in vivo experiments. However, it was suggested that, in in vitro assays, it could lead to the cytotoxicity described by Hellström et al. (1971).

H. Aims of the present investigation

The present experiments were intended to examine the nature of the cellular responses involved in the termination of homograft tolerance by means of the transfer of normal lymphocytes. These cellular responses were to be monitored by following the pattern of reappearance of cells with reactivity directed against tissues of the tolerated type in rats which were formerly tolerant.

It was hoped that examination of the sequence of

reappearance of reactive cells might clarify the mechanism whereby transferred, normal lymphocytes restore the immunological reactivity of tolerant recipients to normality. The alternative mechanisms were envisaged as re-equipment of the lymphoid tissues of the tolerant host by the transferred lymphocytes and their descendants and the re-constitution of the tolerant rat's reactivity by means of its own cells. In the latter case, the pattern of reappearance would be likely to distinguish between the possibilities that the reactive cells had arisen by re-activation of suppressed cells and the generation of new cells.

Finally, it was anticipated that any information obtainable on the mechanism of termination of tolerance might be useful in drawing some conclusions about the cellular mechanism responsible for maintenance of the tolerant state.

Preparation of cell suspensions

Thoracic duct lymphocytes

The thoracic duct was cannulated in the thorax under ether anaesthesia using Cowan's (1959) modification of the method of Hollman, Cain and Grindley (1948).

After the operation the rats were maintained, unanaesthetized, in restraining cages (Hollman et al., 1948) to which a hopper for food and a drinking bottle were attached.

SECTION 2

Materials and Methods

A. Rats

Rats used in these experiments were obtained from several sources. Three inbred Ag-B incompatible colonies of DA (Ag-B⁴), Lewis (Ag-B¹) and Hooded (Ag-B⁵) strains were maintained.

DA and Lewis strain rats were bred by brother-sister mating from pairs obtained from the Walter and Eliza Hall Institute, Melbourne, the colonies having originally been developed by Dr W. K. Silvers. Hooded strain rats were obtained from the C.S.I.R.O. Division of Animal Physiology, Prospect, New South Wales, Australia. They were originally obtained from Glaxo Laboratories, U.K.

When (DA x Lewis) F₁ hybrid or (DA x Hooded) F₁ hybrid or (Lewis x Hooded) F₁ hybrid rats were bred from these colonies, both strains were used as a source of either parent.

B. Preparation of cell suspensions

Thoracic duct lymphocytes

The thoracic duct was cannulated in the abdomen under ether anaesthesia using Gowans' (1959) modification of the method of Bollman, Cain and Grindlay (1948).

After the operation the rats were maintained, unanaesthetized, in restraining cages (Bollman et al., 1948) to which a hopper for food and a drinking bottle were attached.

Lymph from the thoracic duct cannula was collected in sterile flasks at room temperature. Each flask initially contained 5 ml. of medium 199 plus 20 units of heparin/ml (Evans Medical Ltd, Liverpool, U.K.). During the period of lymph collection, rats received an intermittent infusion of Dulbecco's saline containing 2 units of heparin/ml via a femoral vein cannula at the approximate rate of 10 ml/12 hours. When cannulated rats were to remain alive for subsequent experiments, the thoracic duct cannula was gently pulled out of the rat (less than 1 cm) and cut off at skin level, so that the cut end of the cannula was allowed to retract into the dorsal subcutaneous tissues. The femoral vein cannula was cut and knotted at its point of emergence from the skin.

Spleen cells

Rats were killed with ether and the spleen removed by transection of the splenic pedicle. If the rat was required for use in a subsequent experiment, it was anaesthetized with ether, the splenic pedicle was clamped with artery forceps, and the spleen was placed in ice-cold Hank's saline on removal, and a single ligature was tied around the splenic pedicle. Cell suspensions were prepared by disrupting spleens on an 80 mesh, 40 gauge stainless steel grid, the dissociated cells being collected in ice-cold Hank's saline.

Lymph node cells

Rats were killed with ether and the lymph nodes removed. When biopsy of cervical lymph nodes was performed, the rat was anaesthetized with ether, and a midline cervical

incision was made. Several lymph nodes found in the upper part of the cervical region were gently dissected with a cotton swab. The pedicles of these lymph nodes, including blood vessels, were tied and cut in close proximity to the lymph nodes. Cells were expressed from lymph nodes by teasing the tissues with fine forceps in ice-cold Hank's saline.

Bone marrow cells

Rats were killed with ether and both femora and tibiae were removed. Epiphyses were cut off with bone forceps, and the bone marrow was isolated by syringing the marrow cavity with ice-cold Hank's saline containing 2 units of heparin/ml. Bone marrow fragments were then repeatedly pipetted to prepare a cell suspension.

All of the manipulations with spleen cells, lymph node cells and bone marrow cells were carried out at ice temperature. Thoracic duct cells were handled at room temperature. Cell suspensions of various tissues prepared in the above mentioned ways were filtered through gauze before their first centrifugation. All types of cells were washed twice by centrifugation before use. Thoracic duct cells, spleen cells and lymph node cells were centrifuged at 230 x G for 10 minutes and bone marrow cells at 60 x G for 10 minutes. After the first centrifugation, a cell count was made in a haemocytometer.

C. Induction of tolerance

Tolerance was induced by the intravenous injection of newborn rats of one strain with approximately 8×10^7 bone

marrow cells from adult donors of the other strain. The bone marrow cells were suspended in a maximum of 0.15 ml Hank's saline and injected into the retro-orbital veins of the recipients using a tuberculin syringe and 30 gauge needle. In all cases, the injections were given within 24 hr of birth. Approximately 6 wk later, the inoculated animals received a skin graft from the same donor strain as the tolerance-inducing inoculum. Survival of the graft in excellent condition for longer than 50 days was arbitrarily taken as evidence of tolerance.

D. Skin grafting

Skin was removed from the ventral wall of the donor and trimmed free of underlying fat, muscle and panniculus. During placement of grafts, additional skin prepared in this manner was kept in normal saline at 4°C. and was used within 4 hours. A graft bed of approximately 2 x 2.5 cm with an intact panniculus was prepared on the lateral thoracic wall of the recipient. The skin was placed on the bed, anchored with silk sutures, and covered with a sterile surgical gauze dressing which was held in place with an elastic adhesive bandage. Dressings were removed on the 7th day.

E. Determination of survival end point of skin graft

In most cases of long-established (tolerated) skin grafts undergoing rejection in response to the transfer of normal lymphocytes, the destruction of the graft started with small areas, followed by the complete destruction of larger areas. The survival end point of this type of rejection was taken as the day on which more than three quarters of the

graft surface was destroyed. In this case, the first naked-eye evidence of destruction was scored as the beginning of graft destruction.

Some long-established skin grafts exhibited different patterns of destruction. The color of the graft deepened from a light pink through brick-red to brown. Parallelling this color change in the skin graft, it became hard to touch, followed by a firm scab formation. The whole of the graft area appeared to take the same course. In this type of rejection, the day of appearance of hardness in the skin graft was scored as the end survival point. F_1 hybrid strain skin grafts placed on normal parental rats and 2nd grafts on "terminated-tolerant" rats always took this pattern of destruction.

The other pattern of rejection was that initial partial or entire alopecia, sometimes prolonged, was followed by the destruction of graft surface. In this case, the day on which approximately three quarters of the graft surface were destroyed was taken as the end point. This condition was sometimes temporary, with eventual recovery of the graft to a normal, healthy appearance.

F. Production of isoantiserum

Rat isoantisera were prepared by injecting members of one strain of rat with spleen cells from the other strain. Each prospective antiserum donor received an initial intraperitoneal injection of approximately 10^8 spleen cells from a rat of the other strain, suspended in complete Freund's adjuvant. (Difco Laboratories, Detroit, U.S.A.). Two or

three subsequent intraperitoneal injections of spleen cells were given at intervals ranging from 1 wk to 2 wk. Antiserum was prepared from blood taken 7 days after the last injection.

G. Incubation of lymphocytes

1) With isoantiserum

When thoracic duct cells were to be incubated with antisera prior to testing for graft-versus-host activity the following procedure was followed, each cell suspension being incubated with both anti-DA and anti-Lewis sera. 5×10^7 cells suspended in 1 ml of medium 199 were mixed with 1 ml of a 1:2 dilution of the specific antiserum in medium 199. 1 ml of a 1 in 2 dilution of freshly reconstituted lyophilized guinea pig serum (Commonwealth Serum Laboratories, Melbourne, Australia) was added as a source of complement. After incubation at 37°C for 30 min, the cells were counted, washed in Hank's saline to remove antiserum, resuspended at a concentration of $5 \times 10^7/\text{ml}$, and then tested for graft-versus-host activity.

2) With Mitomycin-C

Thoracic duct lymphocytes were resuspended in the appropriate volume of medium 199 containing Mitomycin-C (Sigma Chemical Company, St Louis, U.S.A.) at a concentration of $25 \mu\text{g}/\text{ml}$ and 10% foetal calf serum to give an approximate cell concentration of $10^8/\text{ml}$. Incubation with Mitomycin-C was performed for 30 min at 37°C . Following incubation, lymphocytes were washed twice in Hank's saline before being used further in an experiment.

H. Gamma irradiation

Irradiation was performed at the Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization, Black Mountain, A.C.T., Australia. A 100 curie cobalt 60 (Co^{60}) source was used. Rats were irradiated in rotating metal drums placed 20 cm from the source. Injection of sublethally irradiated rats was carried out within the first 24 hr after irradiation.

I. Popliteal lymph node weight assay

The popliteal lymph node weight assay for graft-versus-host activity of lymphocytes was performed as described by Ford, Burr and Simonsen (1970). 0.1 ml of a suspension of lymphocytes in Hank's saline was injected subcutaneously into the foot-pad of 4 to 7 week-old rats of either sex using a tuberculin syringe and 25 gauge needle. The popliteal lymph node was removed and weighed with an accuracy of 0.1 mg seven days later. When this assay was applied to the sex discriminative assay to be described in Sections 3 and 4, 3 to 4 week-old female (DA x Lewis) F_1 hybrid rats were used as recipients for the preliminary injection of bone marrow cells from male Lewis rats.

J. Histology

Tissues were fixed in formol saline, embedded in paraffin, sectioned at 5μ , and stained with haematoxylin and eosin.

SECTION 3

Preliminary and Control Experiments

A number of experimental observations served as "base lines" for interpreting results of the experiments presented in this thesis. For convenience of access in comparing these control observations with experimental results, the former have been collected in this Section.

A. The assay of graft-versus-host activity

Two forms of assay were used. In one of these, F_1 hybrid rats of from 60 to 80 grams of bodyweight were exposed to 455 rads of gamma irradiation and then injected intravenously with the cell suspension to be tested. Irradiation has been shown to render F_1 hybrid rats susceptible to very small numbers of parental strain cells (Goldschneider and McGregor, 1966). Death of the recipients was used as the end point.

The susceptibility of irradiated F_1 hybrid rats to parental strain cells is shown in Table 1. It will be seen that 5×10^6 thoracic duct lymphocytes from normal DA and Lewis rats were lethal, whereas any number (up to 160×10^6) of lymphocytes from tolerant rats were ineffective. Spleen cells from normal parental rats were less effective in killing irradiated F_1 hybrid recipients. Quantitative studies on the graft-versus-host activity of lymphoid cells from various tissues using popliteal lymph node weight assay has shown that the activity of blood leucocytes is approximately 2 times

TABLE 1

Effect of an intravenous injection of normal or specifically tolerant parental strain lymphoid cells on F_1 hybrid rats.

Donor			Recipient*	
Strain and source of cells	Immunological state	No. cells transferred ($\times 10^{-6}$)	No. inoculated	No. killed [‡]
DA:T.D.L.	Normal	0	7	0
		2	3	0
		5	8	6
		10	2	2
		20	6	5
Lewis:T.D.L.	Normal	5	7	7
		10	4	4
		20	4	4
DA:SP.	Normal	5	5	0
		10	5	1
		20	14	2
		40	9	3
		80	3	2
DA:T.D.L.	Tolerant [‡]	50	1	0
		80	1	0
		100	1	0
		120	2	0
		160	3	0
Lewis:T.D.L.	Tolerant [‡]	5	2	0
		10	2	0
		20	6	0
		40	5	0
		60	1	0
		80	3	0

* Male and female (DA x Lewis) F_1 hybrid rats of 60-80 g body weight. The animals were exposed to 455 rads of whole body Co^{60} -irradiation 1 to 2 hours before injection.

‡ Tolerant DA and Lewis rats which had been bearing (DA x Lewis) F_1 hybrid skin homografts in perfect condition for more than 50 days were used.

‡ Observation period was 50 days after injection.

stronger than that of spleen cells (Yoshida and Osmond, 1971).

The second type of assay of graft-versus-host activity was the popliteal lymph node assay of Ford, Burr and Simonsen (1970). The lymphoid cells to be tested were injected into the foot-pad and the weight of the corresponding popliteal lymph node was measured seven days later. Table 2 shows the popliteal lymph node enlargement produced in F_1 hybrid rats by various doses of parental strain lymphoid cells. An inoculum of 3×10^7 lymphocytes from homograft tolerant animals produced less popliteal lymph node enlargement than did 10^6 normal lymphoid cells.

B. The rejection of tolerated skin homografts following the transfer of normal syngeneic lymphocytes

Doses of less than 25×10^6 syngeneic lymphocytes were insufficient to produce rejection of long-established skin homografts (Table 3). Transfer of 50×10^6 thoracic duct lymphocytes effected rejection in 1 out of 3 tolerant recipients. Transfer of 10^8 lymphocytes resulted in the rejection of tolerated skin 20 days after transfer of thoracic duct lymphocytes, while 4×10^8 thoracic duct lymphocytes invariably caused rejection of long-tolerated F_1 hybrid strain skin grafts within 16 days.

It has been established by Billingham, Brent and Medawar (1956a) that "terminated-tolerant" animals rapidly rejected second grafts placed after rejection of long-established skin grafts occurred. A similar observation was made on re-grafting "terminated-tolerant" rats in the present study (Table 4).

TABLE 2

- * Tolerant rats which had been bearing specific F_1 hybrid skin homografts in perfect condition for more than 50 days were used.
- + Male and female F_1 hybrid rats of 4-7 week-old were used.
- # Number in brackets refers to the number of popliteal lymph nodes of F_1 hybrid recipients examined.

TABLE 2

Popliteal lymph node enlargement produced by the subcutaneous injection of lymphoid cells from normal or specifically tolerant* parental strain rats into the footpads of the appropriate F_1 hybrid rats.

Source of cells	Recipient ⁺	No. of cells transferred ($\times 10^{-6}$)	Popliteal lymph node weight mean \pm standard error (mg)
Normal T.D.L. from DA	(DAXLewis) F_1	0.5	6.9 \pm 0.5 (4) #
		1.0	11.6 \pm 1.6 (4)
		5.0	60.3 \pm 4.9 (4)
		10.0	93.9 \pm 4.4 (4)
Normal L.N.C. from DA	(DAXLewis) F_1	1.0	12.8 \pm 1.4 (4)
		5.0	47.1 \pm 2.3 (4)
		10.0	77.5 \pm 6.9 (4)
		20.0	114.7 \pm 7.8 (4)
Normal SP. from DA	(DAXLewis) F_1	1.0	10.5 \pm 1.2 (4)
		5.0	27.8 \pm 1.9 (4)
		10.0	50.0 \pm 3.3 (4)
		20.0	78.5 \pm 7.4 (4)
Normal L.N.C. from Lewis	(DAXLewis) F_1	5.0	40.7 \pm 1.6 (8)
		10.0	60.1 \pm 3.3 (8)
Normal T.D.L. from DA	(DA \times Hooded) F_1	1.0	15.9 \pm 3.2 (4)
		5.0	51.0 \pm 3.4 (4)
		10.0	77.5 \pm 3.4 (4)
Tolerant T.D.L. from DA tolerant of (Lewis \times Hooded) F_1	(DA \times Hooded) F_1	10.0	7.8 \pm 0.6 (6)
Tolerant L.N.C. from DA tolerant of (Lewis \times Hooded) F_1	(DA \times Hooded) F_1	10.0	8.4 \pm 0.8 (6)
Tolerant L.N.C. from DA tolerant of (Lewis \times Hooded) F_1	(DAXLewis) F_1	5.0	9.7 \pm 0.9 (8)
			10.8 \pm 0.7 (12)
Tolerant T.D.L. from DA tolerant of (DA \times Lewis) F_1	(DAXLewis) F_1	8.0	9.1 \pm 0.7 (4)
		25.0	7.9 \pm 0.5 (4)
		30.0	7.6 \pm 0.6 (4)
Tolerant T.D.L. from Lewis tolerant of (DA \times Lewis) F_1	(DAXLewis) F_1	6.0	10.4 \pm 1.3 (5)
		9.0	10.9 \pm 1.4 (6)
		10.0	8.1 \pm 1.0 (4)
		14.0	6.3 \pm 1.0 (4)

TABLE 3

The ability of various numbers of normal syngeneic T.D.L. to effect rejection of long-established* (tolerated) skin grafts.

No. of cells transferred ($\times 10^{-6}$)	Survival time of F_1 skin
20	N.R.
25	N.R. N.R.
50	32 N.R. ⁺ N.R. ⁺
100	20
200	15 [‡]
300	15 [‡]
400	12 [‡] 12 13 13 13 13 14 15 16 16 16

* Normal syngeneic thoracic duct lymphocytes were injected into tolerant rats which had been bearing tolerated (DA x Lewis) F_1 hybrid skin grafts for more than 50 days.

N.R. Not rejected.

+ Temporary alopecia and linear necrosis along the edges of skin grafts occurred, but grafts finally returned to a normal appearance.

‡ These three tolerant rats were of Lewis strain. All other tolerant rats were of DA strain.

TABLE 4

Prompt rejection of second grafts⁺ on "terminated-tolerant"* rats.

Time of re-grafting after rejection of 1st tolerated grafts (days)	Survival time of regrafted skin
3	8
7	8, 8
24	8, 8
90	7

* 4×10^8 normal DA T.D.L. were injected into DA rats tolerant of (DA x Lewis) F_1 tissues to terminate the tolerant state.

+ Skin for second grafts was obtained from female (DA x Lewis) F_1 hybrids, and grafted on the left side of thoracic wall.

C. The rejection of (DA x Lewis) F₁ hybrid skin homografts on Lewis and DA strain rats

As indicated in Table 5, grafts of skin from (DA x Lewis) F₁ hybrid donors were rapidly rejected after placement on normal Lewis and DA rats.

D. Identification of the origin of lymphocytes mounting a graft-versus-host reaction by the use of female F₁ hybrids sensitized against male specific antigen

In some of the experiments to be reported in Section 4, it was desirable to distinguish the origin of the lymphocytes in a mixture of two syngeneic populations which were responsible for mounting a graft-versus-host response when assayed in the foot-pad of F₁ hybrid rats (Ford, Burr and Simonsen, 1970). This distinction was drawn by performing the foot-pad/popliteal lymph node weight assay in female F₁ hybrid rats which had been sensitized against male specific antigen.

In order to establish an appropriate protocol, female (DA x Lewis) F₁ hybrid rats were immunized, by foot-pad injection, with a range of doses of bone marrow cells from male Lewis rats. At various intervals after this preliminary treatment, these female F₁ hybrid rats were challenged, in the foot-pad, with 10^7 lymph node cells from male Lewis rats. It was found that the combination of a preliminary injection of 2×10^7 bone marrow cells and an interval of 8-16 days before the injection of lymphocytes resulted in a marked suppression of popliteal lymph node enlargement in comparison with that observed in F₁ hybrid rats which had not been pre-treated (Table 6). It was also noted that there was no suppression of

TABLE 5

Rejection of (DA x Lewis) F_1 hybrid skin grafts placed on normal parental strain rats.

Parental strain	Survival time of F_1 hybrid skin (days)
Lewis	7, 7, 8
DA	7, 7, 7, 7, 7, 7, 8

TABLE 6

The effect of pre-treatment of female (DA x Lewis) F_1 hybrid rats* with bone marrow cells from male Lewis donors on the popliteal lymph node enlargement produced by a subsequent injection of Lewis lymph node cells⁺.

Interval between bone marrow cells and lymph node cells (days)	Popliteal lymph node weight (mg) mean \pm standard error	
	Sex of Lewis lymph node cells	
	Male (\uparrow \circ)	Female (\uparrow \circ)
4	47.5 \pm 3.8 (4) [‡]	73.7 \pm 4.2 (4)
8	10.5 \pm 1.3 (4)	51.3 \pm 7.4 (4)
10	12.5 \pm 1.0 (8)	39.9 \pm 3.0 (6)
12	10.7 \pm 0.4 (14)	59.3 \pm 5.3 (14)
16	11.4 \pm 1.1 (4)	93.1 \pm 11.6 (2)

* The footpads of 3-4 week-old female F_1 hybrid rats were injected with 2×10^7 bone marrow cells from male Lewis rats.

+ 10^7 cervical lymph node cells were injected into those footpads which had been injected previously with bone marrow cells from male Lewis rats.

‡ Number in brackets refers to the number of popliteal lymph nodes of F_1 recipients examined. For reference: 10^7 lymph node cells from Lewis strain rats produced 60.1 \pm 3.3 (mg) lymph node enlargement in untreated F_1 hybrid rats (mean of 8 nodes).

the popliteal lymph node response to lymphocytes from female
Lewis donors.

Experimental results

A. The termination of homograft tolerance by means of the
transfer of lymphocytes from normal, syngeneic donors
Tolerance of homografts can be terminated, as
indicated by the rejection of homologous skin grafts, if
lymphocytes from normal, syngeneic donors are transferred to
tolerant hosts. However, the tolerant state, as monitored by
the inability of an animal's lymphocytes to produce graft-
versus-host disease in recipients of the tolerated strain, is
not concurrently reversed by this procedure (Harris, 1977).
The present experiments sought to delineate the pattern of
reappearance of specific graft-versus-host reactivity in a
"terminated-tolerant" rat. The pattern of appearance of
cells with capacity to mount a graft-versus-host reaction
was considered likely to give an indication of the cellular
kinetics of termination of tolerance and, indirectly, of the
cellular basis of tolerance itself. For example, if tolerance
is a consequence of the elimination of those lymphoid cells
which are able to react against the tissues of the tolerated
strain, then the breakage of tolerance will necessitate the
replacement of these cells by cells derived from the injected
normal lymphocytes. Alternatively, if reactive cells exist
in a repressed state in tolerant animals, then breakage of
tolerance may entail a restoration of the reactivity of these
cells. If the former situation applies, reactive cells in the
"terminated-tolerant" rat should be derived solely from the

SECTION 4

Experimental Results

A. The termination of homograft tolerance by means of the transfer of lymphocytes from normal, syngeneic donors

Tolerance of homografts can be terminated, as indicated by the rejection of longstanding skin grafts, if lymphocytes from normal, syngeneic donors are transferred to tolerant hosts. However the tolerant state, as monitored by the inability of an animal's lymphocytes to produce graft-versus-host disease in recipients of the tolerated strain, is not concurrently reversed by this procedure (Elkins, 1972). The present experiments sought to delineate the pattern of reappearance of specific graft-versus-host reactivity in such "terminated-tolerant" rats. The pattern of appearance of cells with capacity to mount a graft-versus-host reaction was considered likely to give an indication of the cellular kinetics of termination of tolerance and, indirectly, of the cellular basis of tolerance itself. For example, if tolerance is a consequence of the elimination of those lymphoid cells which are able to react against the tissues of the tolerated strain, then the breakage of tolerance will necessitate the replacement of these cells by cells derived from the injected normal lymphocytes. Alternatively, if reactive cells exist in a repressed state in tolerant animals, then breakage of tolerance may entail a restoration of the reactivity of these cells. If the former situation applies, reactive cells in the "terminated-tolerant" rat should be derived solely from the

injected cells and should gradually increase in number as these multiply. Alternatively, if the latter applies, the complement of reactive cells in formerly tolerant hosts may increase more rapidly as the result of reappearance of reactivity in pre-existing host cells. In an attempt to differentiate between these possibilities, the appearance of reactive cells in tolerant rats injected with normal syngeneic lymphocytes was examined.

1) The reappearance of reactive cells in tolerant rats following the transfer of lymphocytes from normal, syngeneic donors

The reactivity of lymphocytes from tolerant rats which had received cells from normal syngeneic donors was examined by two methods. In the first, graded numbers of lymphocytes from "terminated-tolerant" donors were transferred to sublethally irradiated (455 rad) F_1 hybrids, weighing 60-80 grams. Such recipients have been shown to be susceptible to graft-versus-host disease produced by the injection of reduced numbers of lymphocytes (Goldschneider and McGregor, 1966). The second technique used to examine the reactivity of the cells from tolerant rats after the termination of the tolerant state was the foot-pad/popliteal lymph node assay of Ford, Burr and Simonsen (1970).

The latter assay entails the injection of small doses of parental strain lymphocytes into the hind foot-pads of 4-7 week-old F_1 hybrids, followed, 7 days later, by examination of the draining popliteal lymph node. This technique appears to be more sensitive than assays based upon killing of F_1

hybrids. However, as it is not established that the two types of assay monitor the same activity of parental strain lymphocytes, both were utilized in the present investigation.

In order to define the distribution with which reactive lymphocytes reappear following the termination of the tolerant state, the activity of cells from different sites was examined. Table 1 of the preliminary experiments section, which should be compared with the results of these experiments, summarizes the effects of transferring thoracic duct lymphocytes from normal and untreated tolerant donors of parental strain to lightly irradiated F_1 hybrid strain rats. Table 7 presents the results of experiments in which thoracic duct lymphocytes were collected from 11 DA rats tolerant of (DA x Lewis) F_1 tissues, at intervals ranging from 2 to 9 days after the intravenous injection of 4×10^8 thoracic duct lymphocytes from normal DA rats. A range of doses of lymphocytes from these "terminated-tolerant" rats were passaged to irradiated (DA x Lewis) F_1 hybrid rats. It will be seen that lymphocytes collected from tolerant rats on the second day after the injection of normal cells, had little capacity to kill F_1 hybrid recipients. However, 20 out of 24 recipients of lymphocytes collected on the third and fourth days died. These included two recipients of 10^7 , three recipients of 2×10^7 and all seven recipients of 4×10^7 lymphocytes. The only survivors were three recipients of 2×10^7 and one recipient of 10^7 cells.

In striking contrast with these results, all eleven F_1 hybrid recipients of lymphocytes collected from tolerant

TABLE 7

GvH activity* of thoracic duct lymphocytes of tolerant DA rats which had been injected with 4×10^8 normal syngeneic thoracic duct lymphocytes.

No. cells transferred ($\times 10^{-6}$)	Days after injection of normal syngeneic TDL (days)								Deaths Total
	2	3	4	5	6	7	8	9	
160	0/2 ⁺	1/1	2/2	1/2	0/2	2/3	2/2	1/1	9/15
80	1/2	2/2	3/3	4/4	0/3	1/5	2/4		13/23
40	0/1	2/2	5/5	2/5	0/3	0/7	2/7	3/3	14/33
20	0/2	1/2	2/4	1/3	0/2	0/5	1/5	2/2	7/25
10	0/1	0/1	2/2	0/1	0/1			0/2	2/8
Deaths/total	1/8	6/8	14/16	8/15	0/11	3/20	7/18	6/8	45/104
No. tolerant donor rats	2	2	4	4	4	4	5	2	

* The G.v.H. activity of cells from inoculated tolerant rats was assessed by injection into irradiated (455 rads) (DA x Lewis) F_1 hybrid rats.

+ Numbers of (DA x Lewis) F_1 hybrid rats killed/total numbers of F_1 hybrid rats injected.

rats on the sixth day after receipt of cells from normal donors survived. This group included two recipients of 1.6×10^8 , three recipients of 8×10^7 and three recipients of 4×10^7 lymphocytes. Lymphocytes collected on the seventh day had a slightly increased capacity to kill F_1 hybrid recipients, but remained markedly inferior to cells collected on the third and fourth days. Thus, although doses of 8×10^7 and 1.6×10^8 lymphocytes were lethal in one out of five and two out of three F_1 hybrid rats respectively, none of the seven recipients of 4×10^7 lymphocytes was killed. There was a further return of reactivity with the passage of time and, by the ninth day, lower doses of lymphocytes were again lethal, all three recipients of 4×10^7 lymphocytes and both of those injected with 2×10^7 being killed.

The lethal capacity of successive collections of lymphocytes from tolerant rats which had previously been injected with normal lymphocytes was examined to determine whether the observations presented in Table 7 could be attributed to modification of the circulatory pattern of lethal lymphocytes as a result of cannulation. The results from Table 7 have been re-arranged in Table 8 to indicate the capacity of lymphocytes collected at different times after thoracic duct cannulation to kill F_1 hybrid recipients. No correlation of the efficacy of lymphocytes with the time of their collection, relative to cannulation, is apparent. Consequently, it is inferred that there had been no significant modification of the pattern of emergence of reactive cells from the thoracic duct as a result of cannulation.

TABLE 8

Effect of thoracic duct drainage on the GvH activity of thoracic duct lymphocytes.

No. cells transferred ($\times 10^{-6}$)	Successive T.D. collection*				Deaths /total
	1st	2nd	3rd	4th	
160	5/8 ⁺	3/5	1/2		9/15
80	7/10	4/8	1/4	1/1	13/23
40	4/12	4/10	3/6	3/5	14/33
20	2/12	2/8	2/3	1/2	7/25
10	1/3	0/2	1/1	0/2	2/8
Deaths/total	19/45	13/33	8/16	5/10	45/104

* Day after starting cannulation

+ Numbers of (DA \times Lewis) F_1 hybrid rats killed/total numbers of F_1 rats injected.

The preceding experiments in which thoracic duct lymphocytes were transferred from tolerant rats at various intervals after their injection with normal lymphocytes indicated that, although large numbers of reactive cells had appeared in the thoracic duct by four days, the frequency of such cells subsequently decreased sharply. The possibility that this decrease reflected emigration of reactive cells from the circulating pool was examined in another group of experiments.

4×10^8 thoracic duct lymphocytes from normal DA rats were injected into each of fifteen DA rats tolerant of (DA x Lewis) F_1 tissues. At various intervals, ranging from 2 to 10 days thereafter, spleens were removed from these tolerant rats and single cell suspensions were prepared. Graded doses of these spleen cell suspensions were injected intravenously into lightly irradiated (DA x Lewis) F_1 hybrid rats, as in the previous experiments.

The results of these experiments are presented in Figure 1. The consequences of transferring a range of doses of cells from the spleen of each individual donor have been grouped together for simplicity. It is apparent that there is considerable variation between tolerant rats in the frequency of occurrence of reactive cells in the spleen despite the standard size of the inoculum of normal DA lymphocytes. There is also some variation in the response of irradiated F_1 hybrids to the receipt of spleen cells from a single tolerant donor. Nevertheless, it is quite clear that there is no evidence of an increase in occurrence of reactive cells in the spleen at a

Fig. 1. G.v.H. activity of spleen cells of tolerant DA rats which had been injected with 4×10^8 normal syngeneic thoracic duct lymphocytes.

		Time after injection of normal syngeneic T.D.L. (days)								
		2	3	4	5	6	7	8	9	10
No. of cells transferred ($\times 10^{-6}$)	320					△				
	240	▲		▲		□		▲		
	160	▲	▲	▲ ●	△ ○	△ ● ■	▲ ○	▲	●	●
	80	△	▲	▲ ▲ ●	▲ ○	△ ● ■	▲ ○	▲ ○	●	●
	40		▲	▲ ○	△ ○	△ ● ■	▲ ○	▲ ○	●	●
	20		▲	▲ ○	△ ○	○ □	▲ ○	○	○	
	10			○	○	●	○	○	○	

G.v.H. activity of cells from inoculated tolerant rats was assessed by injection into irradiated (455 rads) (DA x Lewis) F_1 hybrid rats.

▲ ● ■ (DA x Lewis) F_1 recipients which were killed following the injection of spleen cells from inoculated tolerant rats.

△ ○ □ (DA x Lewis) F_1 recipients which survived for more than 100 days following injection.

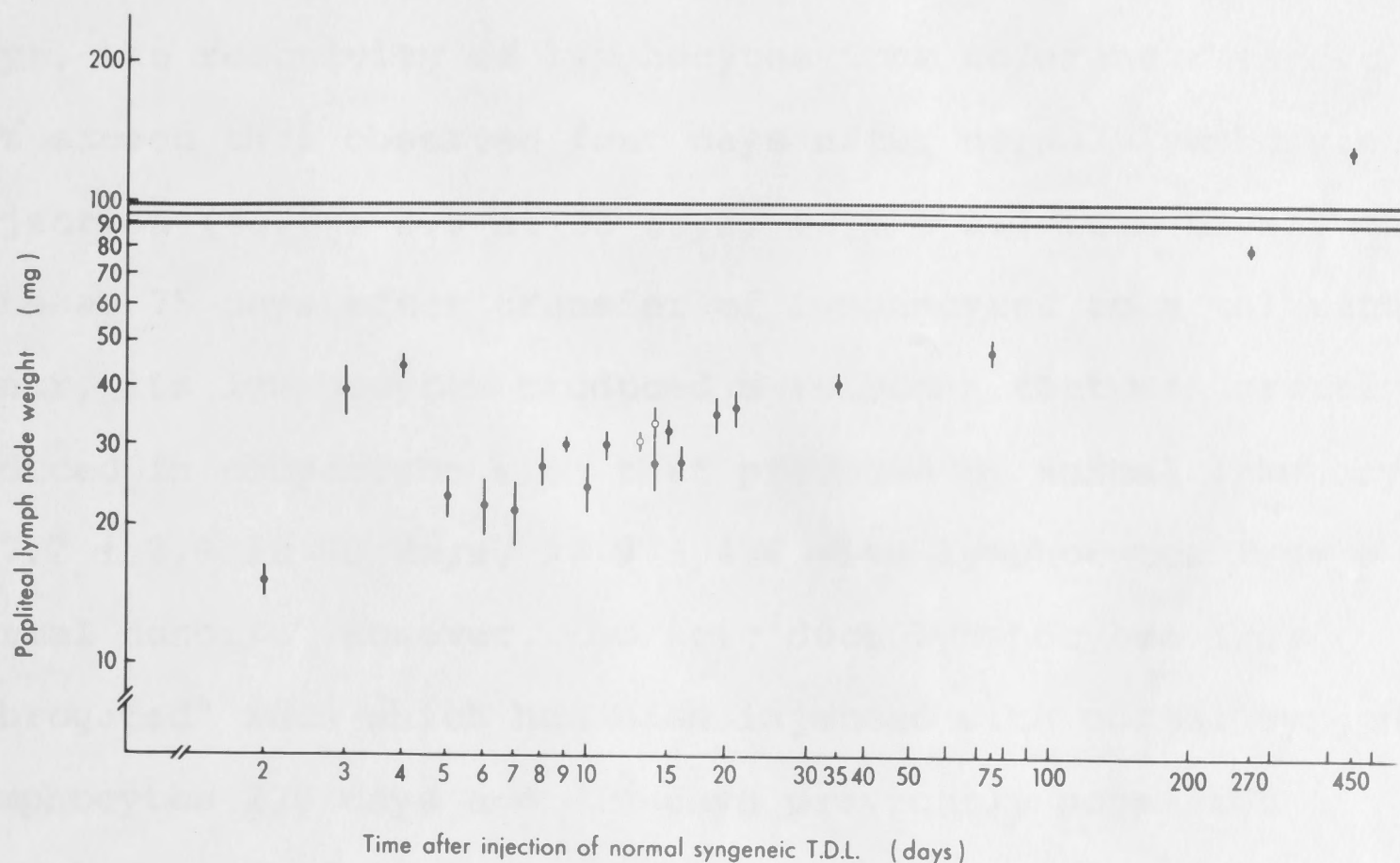
The different symbols used to represent the results obtained with cells collected on a particular day indicate the effects produced by cells from different tolerant donors.

time corresponding to their disappearance from the thoracic duct. On the contrary, the capacity of spleen cells to kill F_1 hybrids also declines markedly from its initial level on the fifth day.

To supplement the preceding results obtained by transferring cells from "terminated-tolerant" rats to lightly irradiated F_1 hybrid recipients the appearance of graft-versus-host activity in such lymphocyte-injected tolerant rats was investigated by means of the popliteal lymph node weight assay (Ford, Burr and Simonsen, 1970). Apart from the use of the different assay technique, the experimental design was identical with that already described. The graft-versus-host activity of thoracic duct lymphocytes, spleen cells and lymph node cells from tolerant rats which had been inoculated with normal syngeneic thoracic duct lymphocytes from 2 to 500 days previously was examined. The results of the transfer of thoracic duct lymphocytes are summarized in Figure 2. It is evident that the graft-versus-host activity of thoracic duct lymphocytes from such rats remained much lower than that of similar cells from normal donors, even 100 days after rejection of tolerated skin grafts. During this period the graft-versus-host activity of these cells failed to attain even 50% of the activity of cells from normal rats.

In view of this prolonged severe depression of graft-versus-host activity, the relatively higher activity of cells collected during the early post-injection periods was particularly interesting. As was the case when the killing of irradiated F_1 hybrids had been used as an assay system, this

Fig. 2. G.v.H. activity of thoracic duct lymphocytes from tolerant DA rats which had been injected with 4×10^8 normal syngeneic thoracic duct lymphocytes.



G.v.H. activity was measured by means of popliteal lymph node weight assay (Ford, Burr and Simonsen 1970). 10^7 cells were transferred. Cells obtained from one, two or three tolerant rats were examined on each day. At least four footpads were injected with cells from each tolerant rat. The results for days 4, 5 and 8 are based on examination of three tolerant rats.

† : mean \pm standard error (mg) of popliteal lymph node enlargement produced by 10^7 thoracic duct lymphocytes from inoculated tolerant rats.

◊ : mean \pm standard error (mg) of popliteal lymph node enlargement produced by 10^7 thoracic duct lymphocytes from tolerant DA recipients of normal DA thoracic duct lymphocytes which had also received 10 daily injections of (DA \times Lewis) F_1 hybrid bone marrow cells.

≡ : mean \pm standard error (mg) of popliteal lymph node enlargement produced by 10^7 thoracic duct lymphocytes from normal DA rats.

augmented reactivity was observed in cells collected on the third and fourth days after transfer of normal lymphocytes to the tolerant rats. Thereafter, there was a reduction of reactivity. Even after rejection of the previously-tolerated skin grafts (which had invariably occurred by the sixteenth day), the reactivity of lymphocytes from tolerant rats did not exceed that observed four days after normal lymphocyte injection (40.3 ± 2.0 at 35 days, 43.9 ± 2.7 at 4 days). As late as 75 days after transfer of lymphocytes to a tolerant donor, its lymphocytes produced a response that was greatly reduced in comparison with that produced by normal lymphocytes (47.2 ± 2.9 at 70 days, 93.9 ± 4.4 with lymphocytes from a normal donor). However, thoracic duct lymphocytes from "abrogated" rats which had been injected with normal syngeneic lymphocytes 270 days and 450 days previously possessed a reactivity of similar strength to that of "normal" lymphocytes. Of particular interest was the completely normal reactivity of lymphocytes from an "abrogated" rat which had received only 5×10^7 normal lymphocytes 450 days previously and then exhibited a significantly prolonged (32 days) graft rejection time. Apart from this rat, all of the tolerant rats used in this experiment were injected with 4×10^8 lymphocytes from normal syngeneic donors. These results with the popliteal lymph node assay resemble those obtained when the ability of lymphocytes to kill irradiated F_1 hybrids was examined during the first 10 days after injection of a tolerant rat with normal lymphocytes. The experiments performed after longer intervals indicated that the recovery of the reactivity

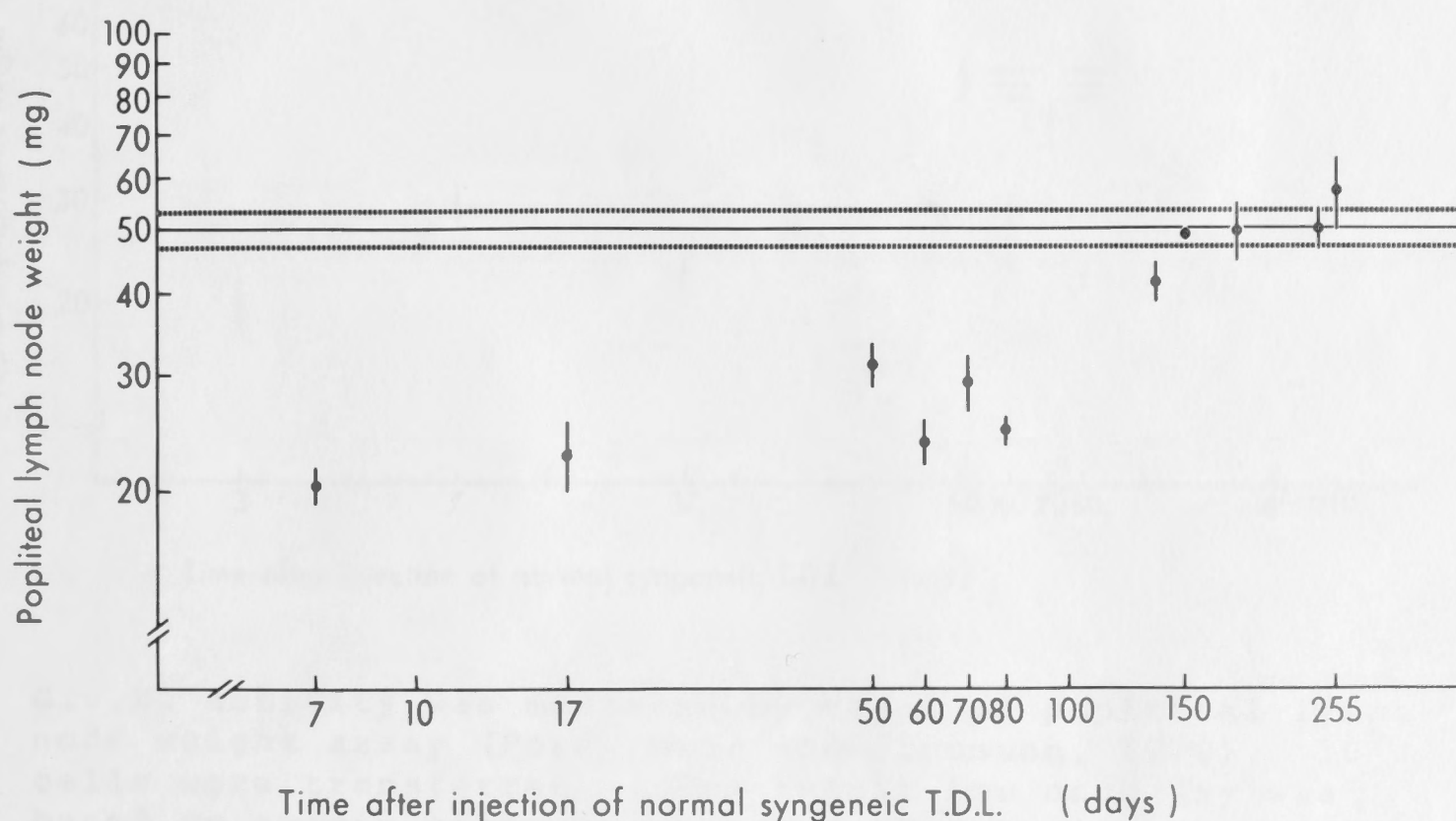
of previously tolerant rats to normal is extremely prolonged.

When the reactivity of spleen and lymph node cells from injected tolerant rats was examined by means of the popliteal lymph node assay, an extremely prolonged period of return to normal levels was again observed. In the case of spleen cells, reactivity did not approach a normal level within 140 days (Figure 3). Similarly, lymph node cells from injected tolerant rats did not manifest normal graft-versus-host activity until after 200 days (Figure 4).

The frequency of occurrence of cells of the tolerated strain in the tissues of a tolerant animal, that is the extent of chimaerism, has been incriminated as influencing the ease with which the tolerant state is terminated following the transfer of normal lymphocytes. However, the nature of this influence has been interpreted in diametrically diverse ways, a high level of chimaerism being claimed both to encourage (Billingham, 1963; Billingham, Silvers and Wilson, 1965; Silvers and Billingham, 1969a) and to retard (Gowans and McGregor, 1965) the rejection of skin grafts by tolerant rats after the injection of normal lymphocytes.

To determine whether the facility with which normal reactivity could be restored following the injection of normal syngeneic lymphocytes was influenced by the frequency of chimaeric cells of the tolerated strain in its tissues, a further experiment was undertaken. Two DA rats, tolerant of (DA x Lewis) F_1 hybrid tissues were injected with 4×10^8 thoracic duct lymphocytes from normal DA donors and then received 10 daily injections of 10^8 bone marrow cells of

Fig. 3. G.v.H. activity of spleen cells from tolerant DA rats which had been injected with 4×10^8 normal syngeneic thoracic duct lymphocytes.

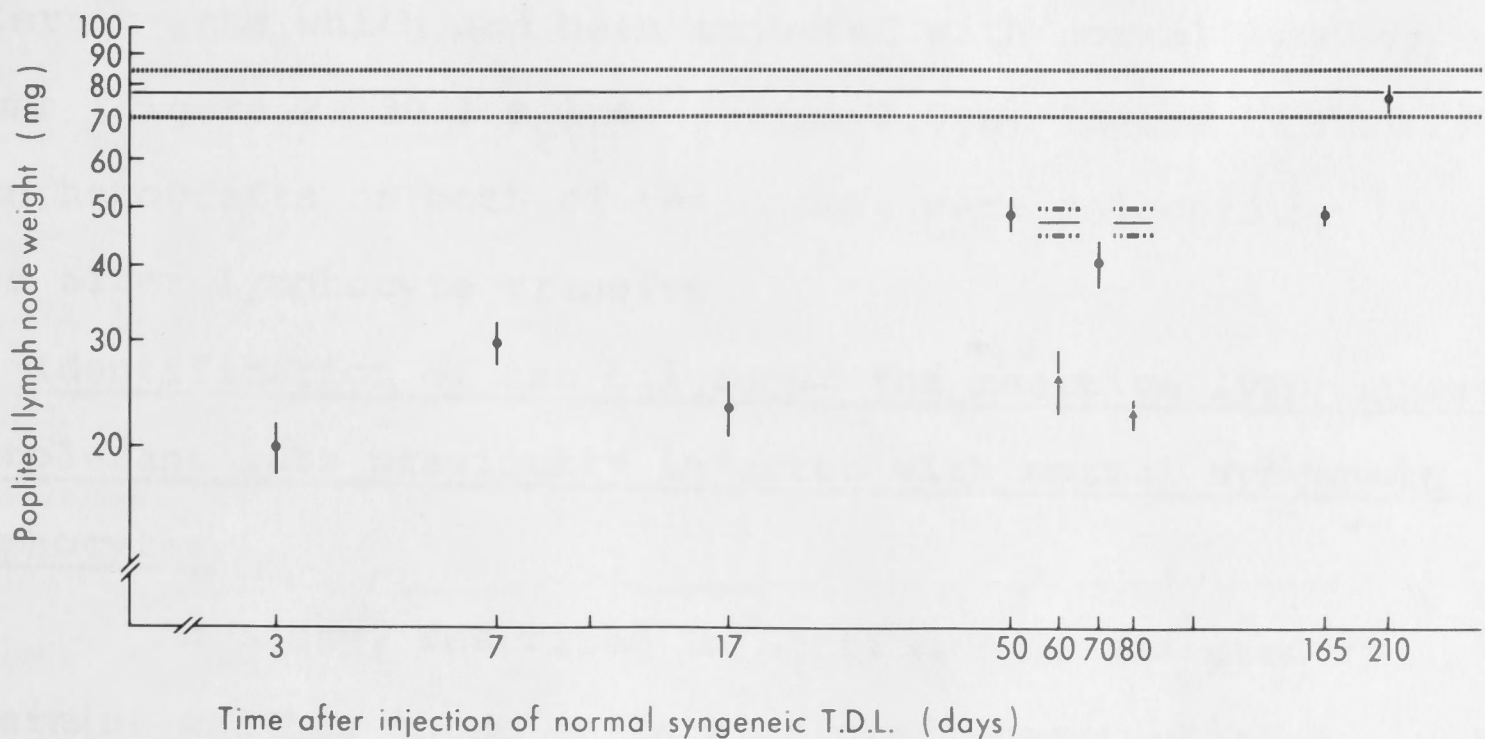


G.v.H. activity was measured by means of popliteal lymph node weight assay (Ford, Burr and Simonsen, 1970). 10^7 cells were transferred. The result for each day was based on examination of one tolerant rat with at least four footpads being injected with cells from each tolerant rat.

† : mean \pm standard error (mg) of popliteal lymph node enlargement produced by 10^7 spleen cells from inoculated tolerant rats.

— : mean \pm standard error (mg) of popliteal lymph node enlargement produced by 10^7 spleen cells from normal DA rats.

Fig. 4. G.v.H. activity of lymph node cells from tolerant DA rats which had been injected with 4×10^8 normal syngeneic thoracic duct lymphocytes.



G.v.H. activity was measured by means of popliteal lymph node weight assay (Ford, Burr and Simonsen, 1970). 10^7 cells were transferred. The result for each day was based on examination of one tolerant rat with at least four footpads being injected with cells from each tolerant rat.

- : mean \pm standard error (mg) of popliteal lymph node enlargement produced by 10^7 lymph node cells from inoculated tolerant rats.
- : mean \pm standard error (mg) of popliteal lymph node enlargement produced by 10^7 lymph node cells from normal DA rats.
- |— : mean \pm standard error (mg) of popliteal lymph node enlargement produced by 5×10^6 lymph node cells from inoculated tolerant rats.
- : mean \pm standard error (mg) of popliteal lymph node enlargement produced by 5×10^6 lymph node cells from normal DA rats.

(DA x Lewis) F_1 origin. The first injection of bone marrow cells was given 24 hours after transfer of lymphocytes. Cannulation of the thoracic duct of the recipient was performed 13 and 14 days later. The lymphocytes from these tolerant recipients displayed reactivity similar to that of tolerant rats which had been injected with normal lymphocytes alone (Figure 2. 30.1 ± 1.4 , 33.1 ± 2.6 at 13 and 14 days). Skin homografts on both of these rats were rejected by 13 days after lymphocyte transfer.

2) Identification of the origin of the reactive lymphocytes in tolerant rats previously injected with normal syngeneic lymphocytes

An assay, described in Section 3-D, was used to determine whether those cells with graft-versus-host activity which appeared in previously tolerant Lewis rats following the injection of lymphocytes from normal Lewis rats were derived from donor or host cells. The assay, which is based on the previous sensitization of female (DA x Lewis) F_1 hybrid rats (to be used in the popliteal lymph node test) against male specific antigen(s) is capable of distinguishing between lymphocytes from male and female donors as the mediators of a graft-versus-host reaction. In order to apply this assay system to distinguishing the activity of donor and host cells, lymphocytes collected from the thoracic duct of one Lewis rat were transferred to a syngeneic rat of the opposite sex tolerant of (DA x Lewis) F_1 hybrid tissues. The latter rat was itself submitted to thoracic duct cannulation and the cells obtained in this manner were injected into the foot-pads of

female (DA x Lewis) F_1 hybrid rats which were either normal or pre-immunized with bone marrow cells from male Lewis rats. Table 9 records the results of applying this assay system to determining the origin of reactive cells appearing in five tolerant Lewis rats as a result of the transfer of lymphocytes from normal Lewis donors of the opposite sex. In four of these experiments [(a) to (d)] the cells collected from the thoracic duct of the tolerant host on the third, fourth and fifth days after injection of "normal" lymphocytes were assayed. It is evident that the majority of the reactive cells in these tolerant hosts were of donor origin. In contrast with this finding, examination of cervical lymph node cells taken from a Lewis rat in which homograft tolerance had been abrogated 20 weeks previously indicated that reactivity against (DA x Lewis) F_1 hybrid tissues was attributable to host-derived cells [experiment (e)].

B. The termination of tolerance by means of cells passaged from "terminated-tolerant" donors

The capacity of thoracic duct lymphocytes from normal DA strain rats to abrogate tolerance of (DA x Lewis) F_1 hybrid skin homografts, when passaged from one tolerant DA strain rat to another, has been investigated. Each member of a group of 11 tolerant DA rats was injected intravenously with 4×10^8 thoracic duct lymphocytes from normal DA donors. As each animal rejected its graft, its thoracic duct was cannulated (approximately 10 days later). The thoracic duct cells collected from each donor over the course of several days, were transferred to a second DA rat tolerant of (DA x Lewis)

TABLE 9

Determination of the origin of G.v.H. reactive cells appearing in tolerant Lewis rats, following the transfer of normal syngeneic T.D.L., by means of the sex discriminative assay.

Sex of normal lymphocyte donor and tolerant host	Time after injection of syngeneic TDL from donor of opposite sex (days)	Source of* cells used for GvH assay	Popliteal lymph node weight (mg) mean \pm standard error#	
			Female (DA x Lewis) F ₁ recipient	
			2 x 10 ⁷ male Lewis B.M.C. treated F ₁ ♀	Untreated F ₁ ♀
T.D.L. from normal ♂ Lewis ↓ tolerant ♀ Lewis	(a) 3	T.D.L.	9.6 \pm 2.3	24.4 \pm 4.6
	(b) 3 4 5	T.D.L.	10.1 \pm 0.7	25.0 \pm 1.1
			10.6 \pm 1.1	27.1 \pm 3.2
			7.8 \pm 1.0	21.6 \pm 0.9
	(c) 3 4	T.D.L.	7.7 \pm 0.6	24.0 \pm 0.9
			8.8 \pm 0.7	29.3 \pm 2.7
T.D.L. from normal ♀ Lewis ↓ tolerant ♂ Lewis	(d) 3 4	T.D.L.	30.9 \pm 0.7 33.0 \pm 4.7	49.4 \pm 5.0 35.6 \pm 2.4
	(e) 150	L.N.C.	21.5 \pm 2.6	53.9 \pm 5.0

* 10⁷ lymphoid cells were injected into the footpads of (DA x Lewis) F₁ hybrid female recipients, approximately 12 days after pre-treatment with 2 x 10⁷ B.M.C. from male Lewis donors.

Four popliteal lymph nodes of female F₁ hybrid recipients were examined.

♂ Male ♀ Female.

F_1 hybrid tissues. Each of these secondary hosts received approximately 10^9 thoracic duct lymphocytes from this source. Following rejection of the skin grafts of the tolerant, secondary hosts, the transfer procedure was repeated with an inoculum of approximately 10^9 lymphocytes from each "terminated-tolerant" secondary host being passaged to a further tolerant DA rat (tertiary recipient).

Subsequently, thoracic duct lymphocytes were collected from those tertiary recipients, which had rejected their grafts and were transferred in similar fashion to other tolerant DA rats. During the course of these passages, the graft-versus-host activity of the lymphoid cells of participating rats was assessed by means of the popliteal lymph node assay of Ford, Burr and Simonsen (1970). To simplify the presentation of the results from these experiments, tolerant animals which received normal thoracic duct lymphocytes (the original inoculum) were designated T_1 and the secondary tolerant hosts which received cells from the T_1 tolerant rats were referred to as T_2 . Similarly, the third group of tolerant hosts was designated T_3 and the fourth ones T_4 . (The experimental protocol is outlined in Figure 5).

1) Prolongation of survival time of skin homografts in successive recipients

As shown in Table 10, all T_1 and T_2 tolerant rats successfully rejected their skin homografts. Skin homografts on T_1 rats were rejected within 16 days (range: 12-16 days) after the transfer of 4×10^8 lymphocytes from normal, syngeneic rats while grafts on T_2 rats were, with one exception,

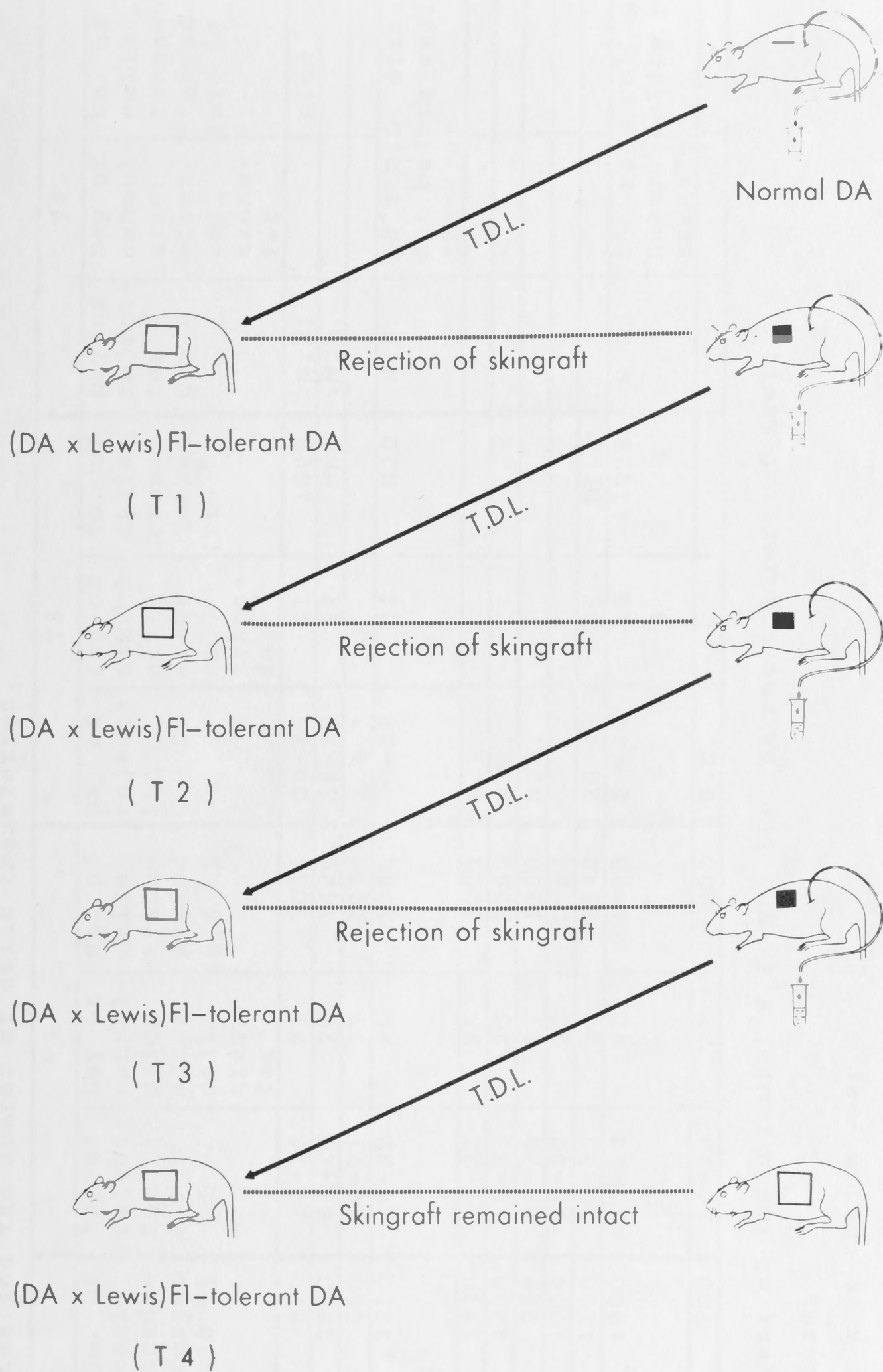


Fig. 5. Experimental design for examination of the passage of tolerance-terminating ability of thoracic duct lymphocytes (see text). An initial injection of 4×10^8 thoracic duct lymphocytes from normal DA rats was given to tolerant rats. When long-established tolerated skin grafts were rejected, following the transfer of 4×10^8 thoracic duct lymphocytes from normal rats, these "terminated-tolerant" rats were subjected to thoracic duct cannulation, generally within 10 days after rejection, and the thoracic duct lymphocytes collected (approximately 10^9) were injected into other tolerant rats. This procedure was repeated until the tolerant rats injected failed to reject their skin grafts.

TABLE 10

Passage of T.D.L. from "terminated-tolerant" rats. Time of skin graft rejection and cannulation of thoracic duct relative to cell transfer and the number of cells transferred.

Stage of passage Tolerant Rat No.	T1			T2			T3			T4		
	Day of rejection C - F [‡]	Day of cannulation after cell transfer	No. of cells transferred (x10 ⁻⁶)	Day of rejection C - F	Day of cannulation after cell transfer	No. of cells transferred (x10 ⁻⁶)	Day of rejection C - F	Day of cannulation after cell transfer	No. of cells transferred (x10 ⁻⁶)	Day of rejection C - F	Day of cannulation after cell transfer	No. of cells transferred (x10 ⁻⁶)
1	9-14	20	810	10-18	21	1030	20-35	41	1020	N.R.		Died
2	11-13	20	1130	11-17	28	1420	16-23	27	960	N.R.		
3	10-13	20	660	14-21	27	1270	N.R.*					
4	12-16	22	1250	15-22	25	1050	20-28	54	820	N.R.	10 ⁹ T.D.L. drained out before cell transfer	
5	12-16	22	1320	17-22	25	1200	N.R.					
6	11-16	22	1230	18-21	25	1250	N.R.		Died			
7	11-15	18	950	23-36	47	1300	N.R.					
8	9-12	20	1000	15-20	26	Died						
9	10-13	34	1200	10-13	20	1140	26-45	48	Died			
10	10-13	34	1000	10-14	20	1490	24-41	48	1025	N.R.	550 rads Co ⁶⁰ exposure before cell transfer	
11	11-15	19	700	16-20	22	400	N.R.					

Nos 1 - 10: DA rats tolerant of (DA x Lewis) F₁; No. 11: DA rat tolerant of Lewis

[‡] C = Commence, F = Finish

* N.R. = Not rejected for more than a year.

rejected within 22 days (range: 13-22 days) after the receipt of approximately 10^9 thoracic duct lymphocytes from "terminated-tolerant" T_1 rats. The exception was the T_2 rat in series No. 7 which did not reject its graft until the 36th day after injection.

In contrast to T_1 and T_2 rats, only five of the ten T_3 rats rejected their tolerated skin homografts (range: 23-45 days) after receiving thoracic duct lymphocytes from "terminated-tolerant" T_2 rats. The other T_3 rats, which failed to reject their tolerated skin homografts within 45 days continued to bear their grafts over a year later.

None of the T_4 rats rejected its skin homograft during the year following the injection of lymphocytes. T_4 rat No. 2 was still bearing its skin graft more than 20 months after being injected. Of the four T_4 rats, two were subjected to procedures intended to deplete the lymphoid tissues of cells before being injected with passaged lymphocytes. Number 4 was depleted of 10^9 lymphocytes by means of drainage from a thoracic duct fistula, while No. 10 received 550 rad of gamma irradiation. In neither case was graft rejection observed after the injection of lymphocytes from the respective T_3 donors.

2) Graft-versus-host reactivity of cells from tolerant recipients of passaged lymphocytes

The graft-versus-host activity of lymphocytes from "terminated-tolerant" rats was examined at the time of passaging these cells to the following generation of tolerant hosts (Table 11). The response to 10^7 cells from both first

TABLE 11

- * G.v.H. activity was measured by means of the popliteal lymph node weight assay (Ford, Burr and Simonsen, 1970). No. of cells transferred was 10^7 .
- § Mean \pm standard error (mg). Four popliteal lymph nodes of F_1 hybrid recipients were examined.
- ‡ Day of cannulation after receiving T.D.L.
- ‡ Day of collection of T.D.L. (relative to cannulation).
- N.T. Not tested.

TABLE 11

G.v.H. activity* of thoracic duct lymphocytes from "terminated-tolerant" rats at the time of their passage to tolerant hosts and of spleen and lymph node cells collected immediately after closure of the thoracic duct fistula (approximately 10^9 thoracic duct lymphocytes drained out).

Stage of passage Tolerant Rat No.	T1	T2	T3
4	N.T.	N.T.	T.D.L. (54) 1st: 27.0 ± 2.6
7	T.D.L. (18) 1st: 39.5 ± 4.1 ± 2nd: 29.5 ± 2.5 S.P. 18.5 ± 2.7 L.N.C. 12.7 ± 2.1	T.D.L. (47) 1st: 31.3 ± 1.6 S.P. 12.0 ± 0.6 L.N.C. 11.5 ± 0.9 2nd: 14.1 ± 1.9	N.T.
8	T.D.L. (20) 1st: 39.7 ± 2.6 S.P. 21.8 ± 1.4 L.N.C. 14.9 ± 1.1 2nd: 31.7 ± 4.9	N.T.	N.T.
9	T.D.L. (34) 1st: 42.4 ± 3.9 S.P. 30.1 ± 1.6 L.N.C. 27.9 ± 2.3 2nd: 35.1 ± 3.9	T.D.L. (20) 1st: 25.7 ± 1.3 2nd: 19.8 ± 1.2	S.P. (T3 rat died during (48) 8.1 ± 1.5 operation. GvH ac- tivity was measur- L.N.C. ed before draining 9.0 ± 2.3 out T.D.L.)
10	T.D.L. (34) 1st: 43.0 ± 3.5 S.P. 12.5 ± 1.6 L.N.C. 21.3 ± 2.0 2nd: 36.8 ± 3.0	T.D.L. (20) 1st: 30.0 ± 2.3 2nd: 18.7 ± 1.8	T.D.L. (48) 1st: 11.6 ± 1.6
11	T.D.L. (19) 1st: 33.3 ± 2.6 2nd: 32.8 ± 1.7	T.D.L. (22) 1st: 31.6 ± 1.2	N.T.

and second day collections of lymphocytes was examined by use of the popliteal lymph node assay of Ford, Burr and Simonsen (1970). As expected, the reactivity of cells from T_1 rats exceeded that of cells from T_2 rats, except in the case of experiment No. 11. The lack of correlation between the activity of passaged thoracic duct lymphocytes as measured by the popliteal lymph node assay and the capacity of these cells to effect termination of tolerance is notable.

There appeared to be little difference in reactivity between lymphocytes collected from the four T_2 rats Nos 7, 9, 10 and 11. Failure of rejection of its skin graft by the T_3 rat in experiment No. 11 is likely to have been due to the reduced numbers of lymphocytes transferred (4×10^8 thoracic duct lymphocytes). However, the failure of rejection of the T_3 graft in experiment No. 7 is inexplicable. Similarly, the failure of a large inoculum of lymphocytes from T_3 in experiment No. 4 to terminate tolerance in the T_4 rat is surprising in view of the extent of graft-versus-host activity in the passaged lymphocytes. Examination of the reactivity of spleen and lymph node cells from "terminated-tolerant" rats showed that even after draining off about 10^9 thoracic duct lymphocytes, spleen and lymph node cells still maintained weak, but significant, levels of activity (Table 11).

3) Comparison of the graft-versus-host activity of lymphoid cells from "terminated-tolerant" rats and rats in which tolerance was not terminated

The graft-versus-host activity of lymphoid cells from rats which had successfully rejected their tolerated skin

homografts was examined at intervals thereafter (Table 12(a)). As was also found in Section 4A this activity remained depressed below normal levels for prolonged periods. It was found that not only T_1 , but also T_2 and T_3 rats had regained normal values of graft-versus-host activity when examined some 12 months after receipt of passaged lymphocytes.

In contrast, reactivity of lymphoid cells from "non-terminated" rats which had failed to reject their tolerated grafts following transfer of lymphocytes remained at a surprisingly low level a year later. No return of normal activity was observed (Table 12(b)).

C. Termination of homograft tolerance by means of isoantiserum

The results of earlier experiments in which lymphocytes were transferred from normal, syngeneic rats to terminate the tolerant state suggested that, although the transferred cells were responsible for graft rejection, the cells responsible for immunologically re-equipping the formerly-tolerant rat were of host origin. If this is so, the pattern of re-appearance of reactive cells in "terminated-tolerant" rats after graft rejection should be similar irrespective of whether normal lymphocytes were transferred to effect termination. This possibility was examined by injecting several tolerant rats with antiserum with the specificity appropriate to produce graft rejection. That antisera can terminate tolerance of homografts was demonstrated by Lubaroff and Silvers (1970) and Hasek et al. (Hasek, Skamene, Karakoz, Chutná, Nouza, Bubeník, Sovová, Nemec and Jonák, 1968).

In the present experiments, three (DA x Lewis) F_1

TABLE 12 (a)

GvH activity* of spleen and lymph node cells from "terminated-tolerant" rats.

Passaged Tolerant Rat No.	T1	T2	T3
No. 1	<u>16.5 months</u> after draining out 810×10^6 TDL L.N.C.: $78.8 \pm 4.3^{\ddagger}$	<u>15.5 months</u> after draining out 1030×10^6 TDL L.N.C.: 81.2 ± 5.2	N.T.
No. 2	<u>11 months</u> after draining out 1130×10^6 TDL SP: 79.4 ± 1.5 L.N.C.: 78.4 ± 4.0	<u>10.5 months</u> after draining draining out 1420×10^6 TDL SP: 35.5 ± 1.8 <u>12 months</u> after draining out 1420×10^6 TDL L.N.C.: 73.7 ± 5.0	<u>9.5 months</u> after draining draining out 960×10^6 TDL SP: 43.2 ± 3.0 L.N.C.: 66.7 ± 2.3

* G.v.H. activity was measured by means of the popliteal lymph node weight assay (Ford, Burr and Simonsen, 1970). No. of cells transferred was 10^7 .

\ddagger Mean \pm standard error (mg). Six to eight popliteal lymph nodes of F_1 hybrid recipients were examined.
N.T. Not tested.

TABLE 12 (b)

- * G.v.H. activity was measured by means of the popliteal lymph node weight assay (Ford, Burr and Simonsen, 1970). No. of cells transferred was 10^7 .
- ‡ Mean \pm standard error (mg). Six to eight popliteal lymph nodes of F_1 hybrid recipients were examined.
- N.T. Not tested.

TABLE 12 (b)

GvH activity* of lymphoid cells from "non-terminated-tolerant" rats.

Passaged Tolerant Rat No.	T3	T4
No. 1	N.T.	140 days after receiving 1020×10^6 T.D.L. T.D.L.: 15.3 ± 1.5
No. 2	N.T.	12 months after receiving 960×10^6 T.D.L. L.N.C.: 11.0 ± 1.1 20 months after receiving 960×10^6 T.D.L. SP.: 9.7 ± 0.6
No. 4	N.T.	12 months after receiving 820×10^6 T.D.L. SP.: 12.4 ± 0.9
No. 7	9 months after receiving 1300×10^6 T.D.L. SP. $11.0 \pm 0.9^\ddagger$	N.T.

hybrid-tolerant Lewis rats were each injected with 10 ml of hyperimmune anti DA serum prepared in Lewis rats as indicated in Materials and Methods. The schedule of administration of antiserum is outlined in Fig. 6.

The long established (DA x Lewis) F_1 hybrid skin grafts were rejected in 2 out of the 3 tolerant rats, as indicated in Table 13, while the third remained intact. To ascertain whether graft-versus-host reactivity against F_1 hybrid tissues had reappeared in these animals, cells obtained by cervical lymph node biopsy were assayed using the test of Ford et al. (Ford, Burr and Simonsen, 1970). The results resembled those obtained following termination of the tolerant state with syngeneic lymphocytes. When examined some time after graft rejection had occurred, graft-versus-host reactivity had partially returned towards that of normal (non-tolerant) rats. However in the third rat, which had received similar treatment with antiserum but had failed to reject its skin graft, the cervical lymph node cells remained unreactive.

D. The partial abrogation of homograft tolerance following the transfer of semi-allogeneic lymphocytes

The experiments of Section 4A suggested that, following the transfer of normal, syngeneic lymphocytes to homograft-tolerant rats, graft rejection was attributable to the activity of the transferred lymphocytes whereas the restoration of specific reactivity in the formerly-tolerant animal depended upon the appearance of cells of host origin. The present experiments were intended to test this hypothesis.

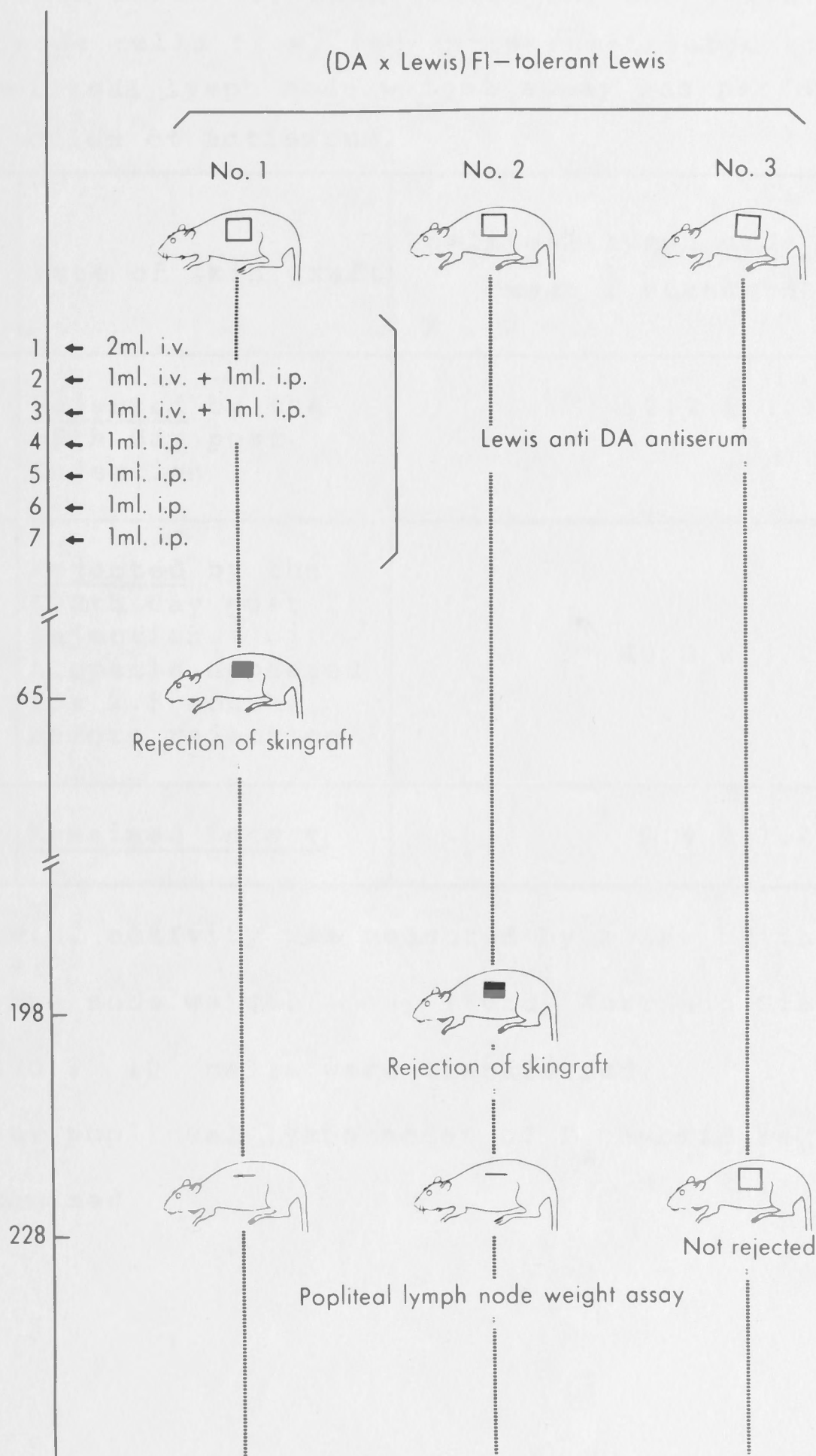


Fig. 6. Lewis rats tolerant of (DA x Lewis) F₁ tissues were injected with undiluted Lewis anti DA isoantiserum for seven successive days, the total volume of isoantiserum administered being 10 ml. 228 days after injection of serum, 10^7 cervical lymph node cells from these tolerant Lewis rats were injected into the footpads of (DA x Lewis) F₁ hybrid rats to test their graft-versus-host activity.

TABLE 13

Fate of (DA x Lewis) F_1 skin grafts on, and G.v.H. activity* of lymph node cells from, iso-antiserum-treated tolerant Lewis rats. Popliteal lymph node weight assay was performed 228 days after injection of antiserum.

Tolerant Rat No.	Fate of skin graft	Popliteal lymph node weight (mg) mean \pm standard error ⁺
1	<u>Rejected</u> by the 65th day post injection	52.7 \pm 0.9
2	<u>Rejected</u> by the 198th day post injection. Alopecia appeared for 2.5 months before rejection	40.3 \pm 4.2
3	<u>Remained intact</u>	9.4 \pm 1.4

* G.v.H. activity was measured by means of the popliteal lymph node weight assay (Ford, Burr and Simonsen, 1970). 10^7 cells were transferred.

+ Four popliteal lymph nodes of F_1 hybrid recipients were examined.

The protocol was based on one described by Silvers and Billingham (1970) and entailed the preparation of DA rats tolerant of (Lewis x Hooded) F_1 hybrid strain tissues (Figure 7). Each of five such DA rats received grafts of skin from Hooded, Lewis and (Lewis x Hooded) F_1 hybrid donors. Subsequently, these tolerant rats were each injected intravenously with 10^9 thoracic duct lymphocytes collected from (DA x Lewis) F_1 hybrid rats which had been specifically sensitized against tissues of Hooded strain rats. (Sensitization was achieved by the placement of skin grafts 14 days, and the injection of spleen cells 7 days, before thoracic duct cannulation).

Both the Hooded and (Lewis x Hooded) F_1 hybrid skin grafts were rejected by all 5 rats in this experiment within 12 days of receipt of Hooded-sensitized (DA x Lewis) F_1 thoracic duct lymphocytes. Lewis strain skin grafts remained intact, indefinitely (in excess of 10 months). The reactivity of cells obtained from these injected tolerant rats by means of cervical lymph node biopsy was assayed in both (DA x Hooded) F_1 hybrids and (DA x Lewis) F_1 hybrids by the popliteal lymph node test of Ford, Burr and Simonsen (1970). As would be anticipated from the results of Section 4A, the graft-versus-host reactivity against (DA x Hooded) F_1 hybrid rats of lymphoid cells from these rats in which tolerance of Hooded strain tissues had been terminated remained close to the level observed in untreated tolerant rats during the first month after rejection of Hooded and (Lewis x Hooded) F_1 hybrid grafts. Reactivity against (DA x Lewis) F_1 hybrid rats

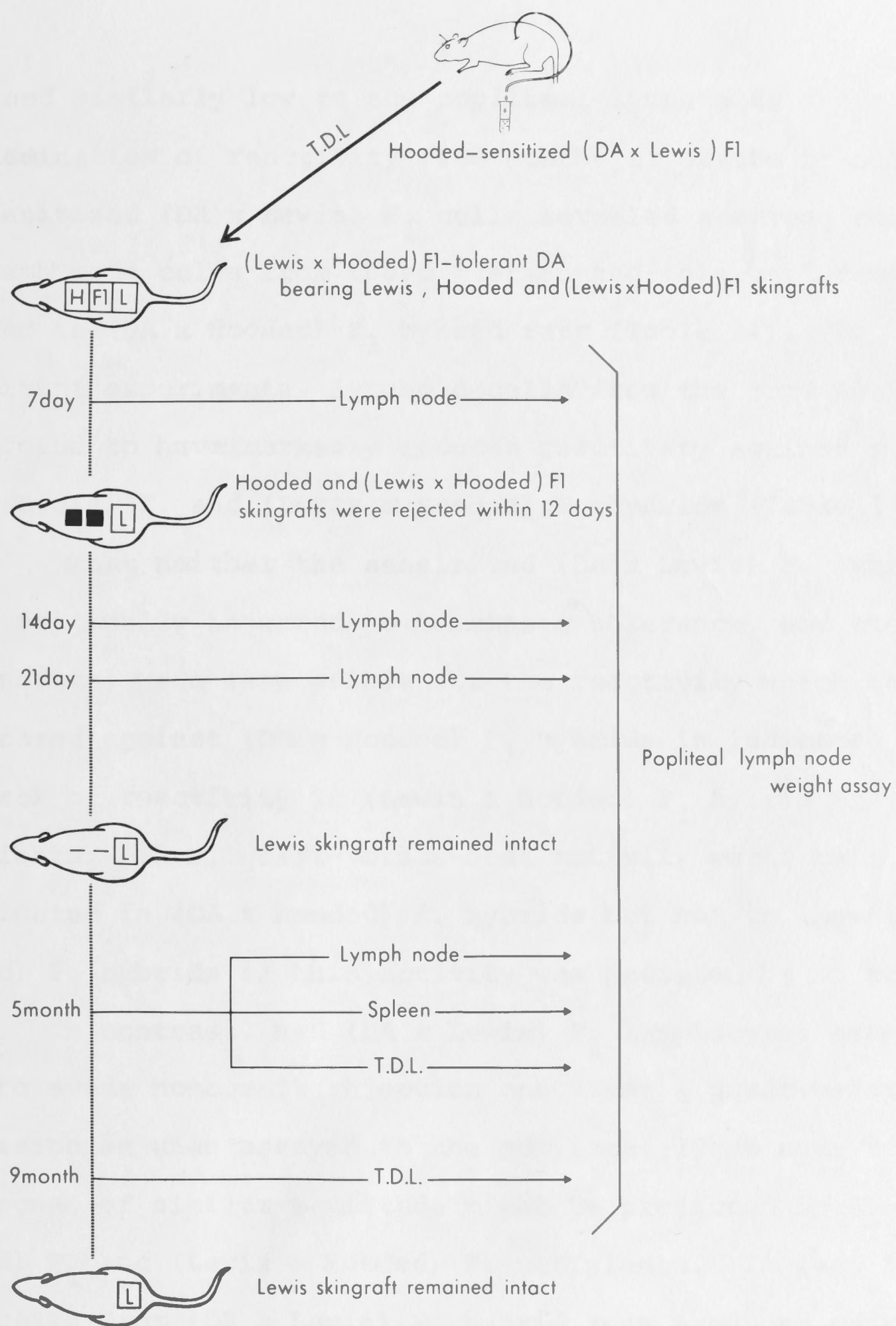


Fig. 7. DA rats tolerant of (Lewis x Hooded) F₁ tissues were injected with 10^9 thoracic duct lymphocytes from (DA x Lewis) F₁ hybrid rats previously sensitized against Hooded tissues. At various times after this injection of cells, the graft-versus-host activity of lymphoid cells from inoculated DA rats was measured by means of the popliteal lymph node weight assay

remained similarly low in the popliteal lymph node assay. Re-examination of reactivity five months after the transfer of sensitized (DA x Lewis) F_1 cells revealed a strong response if lymph node cells from these "terminated-tolerant" rats were assayed in (DA x Hooded) F_1 hybrid rats (Table 14). In concurrent experiments, lymphoid cells from the same source were found to have markedly reduced reactivity against (DA x Lewis) F_1 and (Lewis x Hooded) F_1 hybrids (Table 14).

That neither the sensitized (DA x Lewis) F_1 lymphocytes originally injected to terminate tolerance, nor their descendants, were responsible for the reactivity which had reappeared against (DA x Hooded) F_1 hybrids is indicated by the lack of reactivity in (Lewis x Hooded) F_1 hybrid recipients. Thus, graft-versus-host activity would be anticipated in (DA x Hooded) F_1 hybrids but not in (Lewis x Hooded) F_1 hybrids if this activity was mediated by DA strain cells. In contrast, had (DA x Lewis) F_1 lymphocytes been able to evade homograft rejection and mount a graft-versus-host response when assayed in the popliteal lymph node test, a response of similar magnitude might be predicted in (DA x Hooded) F_1 and (Lewis x Hooded) F_1 recipients. In fact lymph node cells from (DA x Lewis) F_1 hybrid rats produced only a marginal degree of lymph node enlargement in (DA x Hooded) F_1 recipients when administered in twice the dose of cells from "terminated-tolerant" DA rats tested (Table 14). The slight enlargement of the popliteal lymph nodes of (DA x Hooded) F_1 recipients of 10^7 (DA x Lewis) F_1 lymph node cells may reflect sensitization of (DA x Hooded) F_1 recipients by the foot-pad

TABLE 14

* G.v.H. activity was measured by means of the popliteal lymph node weight assay (Ford, Burr and Simonsen, 1970).

+ Number in brackets refers to the number of popliteal lymph nodes of F_1 hybrid recipients examined.

N.T. Not tested.

For reference: 10^7 L.N.C. from (DA x Lewis) F_1 hybrid rats produced 13.0 ± 0.7 (mg) lymph node enlargement in (DA x Hooded) F_1 hybrid rats (mean of 4 nodes).

TABLE 14

G.v.H. activity* of lymphoid cells from (Lewis x Hooded) F_1 -tolerant DA rats which had received 10^9 T.D.L. from Hooded-sensitized (DA x Lewis) F_1 hybrid rats.

Time after transfer of T.D.L.	Source and no. of cells transferred ($\times 10^{-6}$)	Popliteal lymph node weight (mg) mean \pm standard error		
		F_1 hybrid recipients		
		(DA X Hooded) F_1	(DA x Lewis) F_1	(Lewis x Hooded) F_1
7 day	L.N.C.: 5	8.8 \pm 0.4 (6) ⁺	6.6 \pm 0.3 (6)	N.T.
14 day	L.N.C.: 5	13.6 \pm 1.6 (4)	8.0 \pm 1.3 (4)	N.T.
21 day	L.N.C.: 5	14.4 \pm 1.2 (4)	10.3 \pm 0.3 (4)	N.T.
5 month	L.N.C.: 5	30.8 \pm 2.4 (6)	6.2 \pm 1.2 (4)	8.2 \pm 0.7 (4)
5 month	SP.: 10	33.7 \pm 0.9 (8)	9.7 \pm 1.1 (6)	8.7 \pm 0.7 (6)
5.5 month	T.D.L.: 10	64.6 \pm 1.3 (6)	20.4 \pm 1.2 (6)	9.0 \pm 0.3 (6)
9 month	T.D.L.: 10	65.5 \pm 2.1 (8)	18.2 \pm 1.1 (4)	N.T.

injection of (DA x Lewis) F_1 cells.

None of these "terminated-tolerant" DA rats has rejected its Lewis skin graft more than 10 months after the transfer of sensitized (DA x Lewis) cells. Considered together with the failure of lymphoid cells from these rats to mount a graft-versus-host reaction in (DA x Lewis) F_1 hybrid recipients, this skin graft retention indicates that, although tolerance of Hooded-strain tissues has been terminated, these DA rats remain fully tolerant of Lewis strain tissues.

E. The production of graft-versus-host disease in homograft-tolerant rats and the modification of its course

It has been reported that the injection of a homograft-tolerant animal with lymphoid cells from a donor of the tolerated strain results in a graft-versus-host reaction which may be lethal (Billingham and Silvers, 1961b; Martinez, Smith and Good, 1961; Stastny, Stemberge and Ziff, 1963). This phenomenon of graft-versus-host reaction mediated by cells towards which the host has been rendered tolerant provides a means for examining the cellular interactions involved in the termination of homograft tolerance following the transfer of normally reactive lymphocytes. Thus, by employing tolerant rats which have received different combinations of tolerance-inducing inocula and skin grafts, it is possible to select an inoculum of tolerated, allogeneic lymphocytes capable of attacking either the skin graft or the chimaeric cells responsible for the maintenance of the tolerant state, or both of these tissues. By such manipulations, it was hoped to examine the influence exerted by a tolerant host on the rejection of

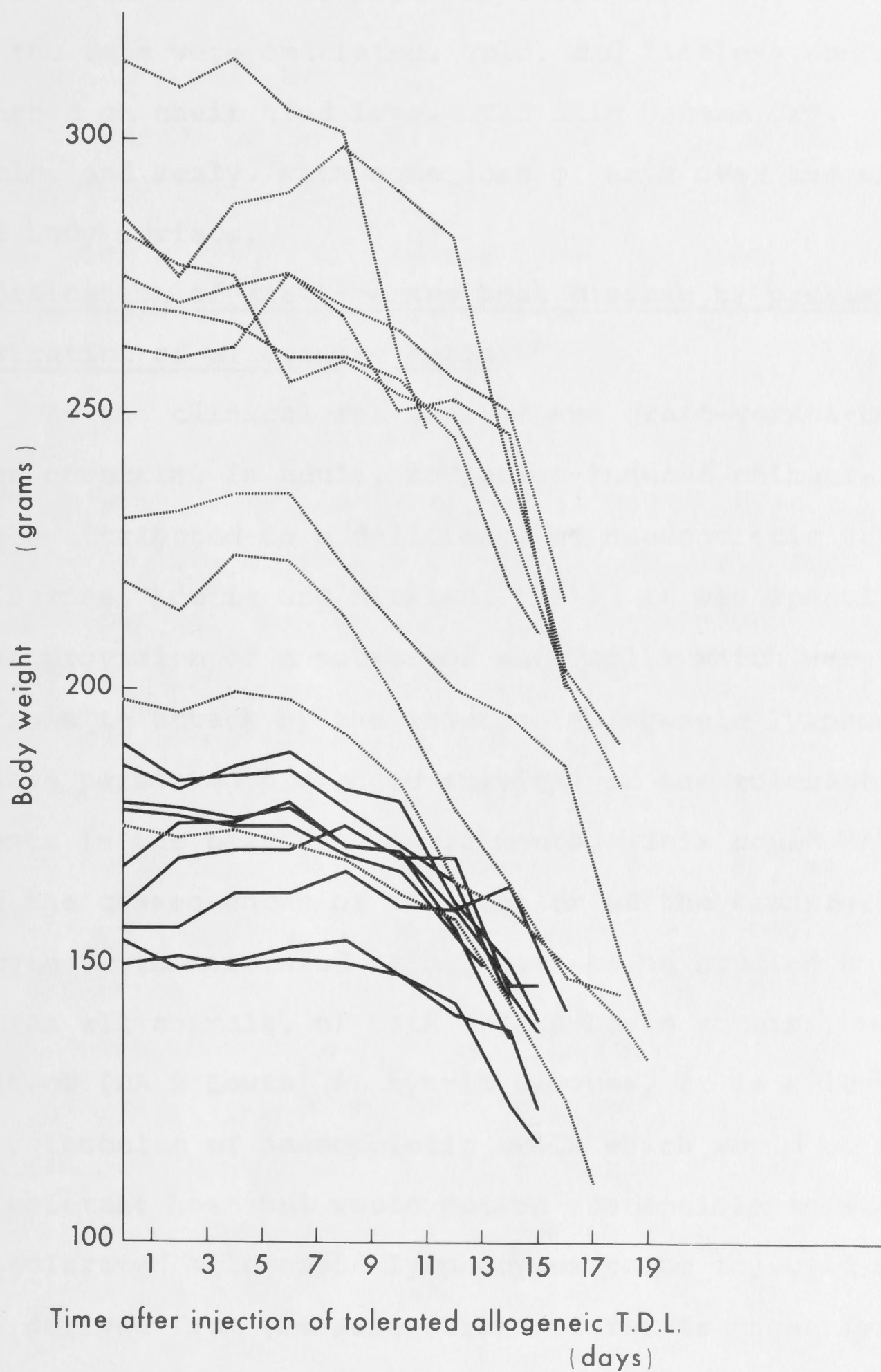
a skin graft by injected, normal lymphocytes and also the relationship of loss of lymphoid tissue chimaerism to skin graft rejection. As a pre-requisite to investigating this aspect of skin graft rejection, the consequences of administering various inocula of tolerated, allogeneic lymphoid cells to tolerant rats were examined.

1) Graft-versus-host disease following the transfer of tolerated, allogeneic lymphocytes alone

Two groups of adult homograft tolerant rats were injected intravenously with allogeneic, but tolerated, thoracic duct lymphocytes. Each rat received 4×10^8 cells in a single inoculum. In the first group, nine DA rats tolerant of (DA x Lewis) F_1 hybrid tissues received lymphocytes from Lewis rats. All of these recipients died between 13 to 16 days after injection. In a similar experiment, ten Lewis rats, also tolerant of (DA x Lewis) F_1 hybrid tissues were injected with lymphocytes from DA donors as a result of which death uniformly occurred between 11 and 19 days later. The behaviour of skin grafts on these tolerant rats will be described in a following section.

Following the injection of tolerated, allogeneic lymphocytes, rats remained clinically well for the first seven days. Around 8 days the first signs of disease appeared and consisted of marked erythema and swelling of the ears, and sometimes of snout and paws. The onset of erythema marked the beginning of a progressive loss of body weight (Figure 8) which, in the last few days of life, became precipitous. Closure of the eyes as a result of the swelling of the palpebral tissues,

Fig. 8. Weight changes of tolerant rats following the receipt of tolerated, allogeneic thoracic duct lymphocytes.



————— DA rats tolerant of (DA x Lewis) F₁ hybrid tissues in receipt of 4×10^8 Lewis thoracic duct lymphocytes.

..... Lewis rats tolerant of (DA x Lewis) F₁ hybrid tissues in receipt of 4×10^8 DA thoracic duct lymphocytes.

All nineteen rats died within 19 days after injection of cells.

and ulceration around the mouth appeared next. In the terminal stages the rats were emaciated, cold, and listless and often sat hunched on their hind legs. The skin became dry, inelastic, and scaly, with some loss of hair over the whole ventral body surface.

2) Modification of graft-versus-host disease by preliminary administration of allogeneic cells

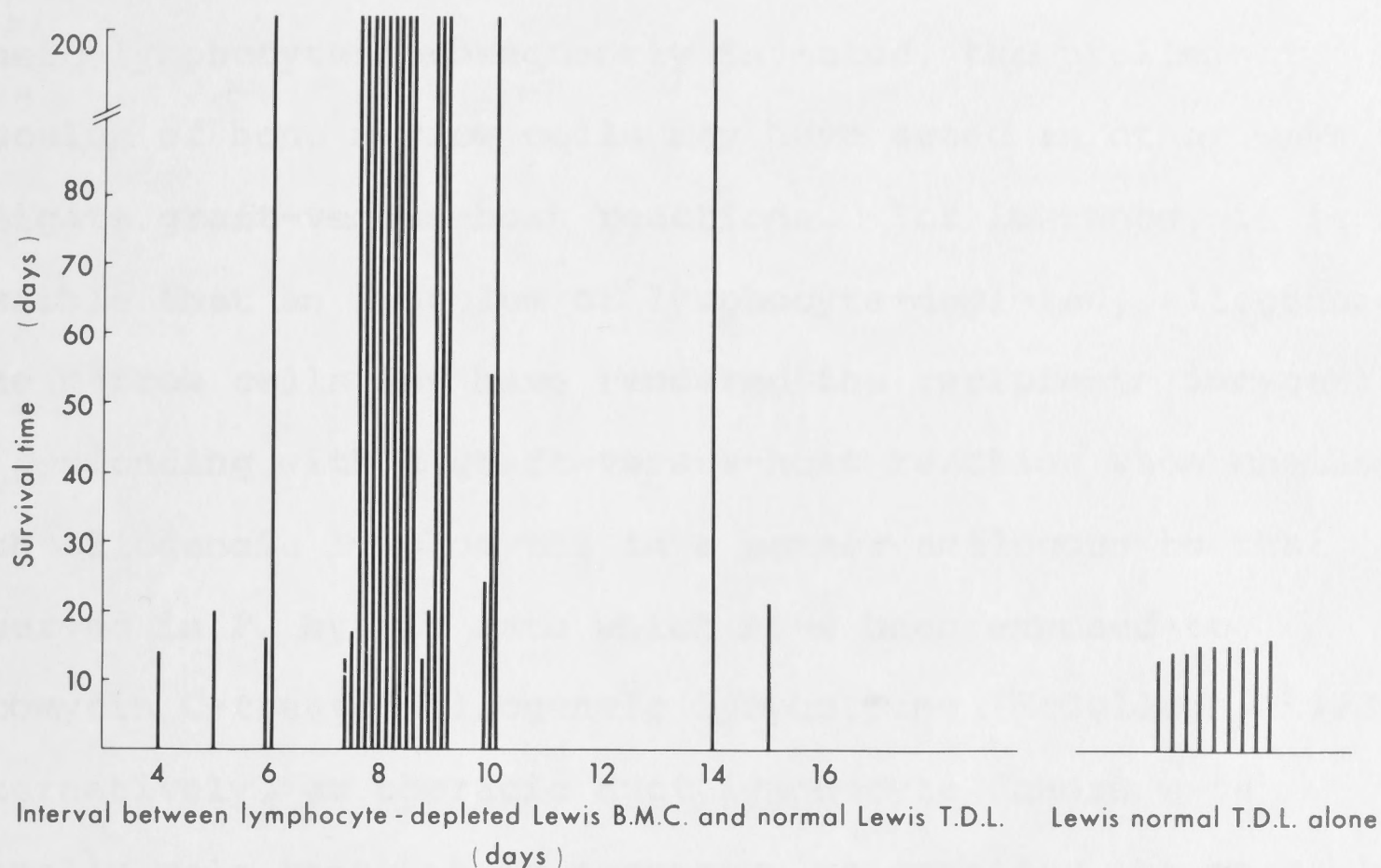
As the clinical features of the graft-versus-host reaction occurring in adult, radiation-induced chimaeras have been attributed to a deficiency of haemopoietic stem cells (Barnes, Loutit and Micklem, 1961), it was speculated that the provision of a source of such cells which were not susceptible to attack by the injected allogeneic lymphocytes might have permitted prolonged survival of the tolerant recipients in the preceding experiments. This could have enabled the consequences of interaction of the transferred lymphocytes with tolerated skin grafts to be studied more fully. As all animals, of both DA and Lewis strains, were tolerant of (DA x Lewis) F_1 hybrid tissues, it is evident that the only inoculum of haemopoietic cells which would be accepted by the tolerant host but would not be susceptible to attack by the tolerated allogeneic lymphocytes to be injected would be one derived from the same strain of rat as these lymphocytes. Accordingly the influence of a preliminary inoculum of 2×10^8 bone marrow cells was studied. In the case of (DA x Lewis) F_1 hybrid-tolerant DA rats, such an inoculum of bone marrow cells from Lewis strain donors preceded the injection of Lewis strain lymphocytes, whilst in contrast, tolerant Lewis

rats received bone marrow cells from DA rats followed by lymphocytes from a similar source. In order to avoid initiating a graft-versus-host reaction with the preliminary inoculum of haemopoietic cells, bone marrow was collected from donors which had been subjected to several days drainage from a thoracic duct fistula to reduce the circulating lymphocyte content.

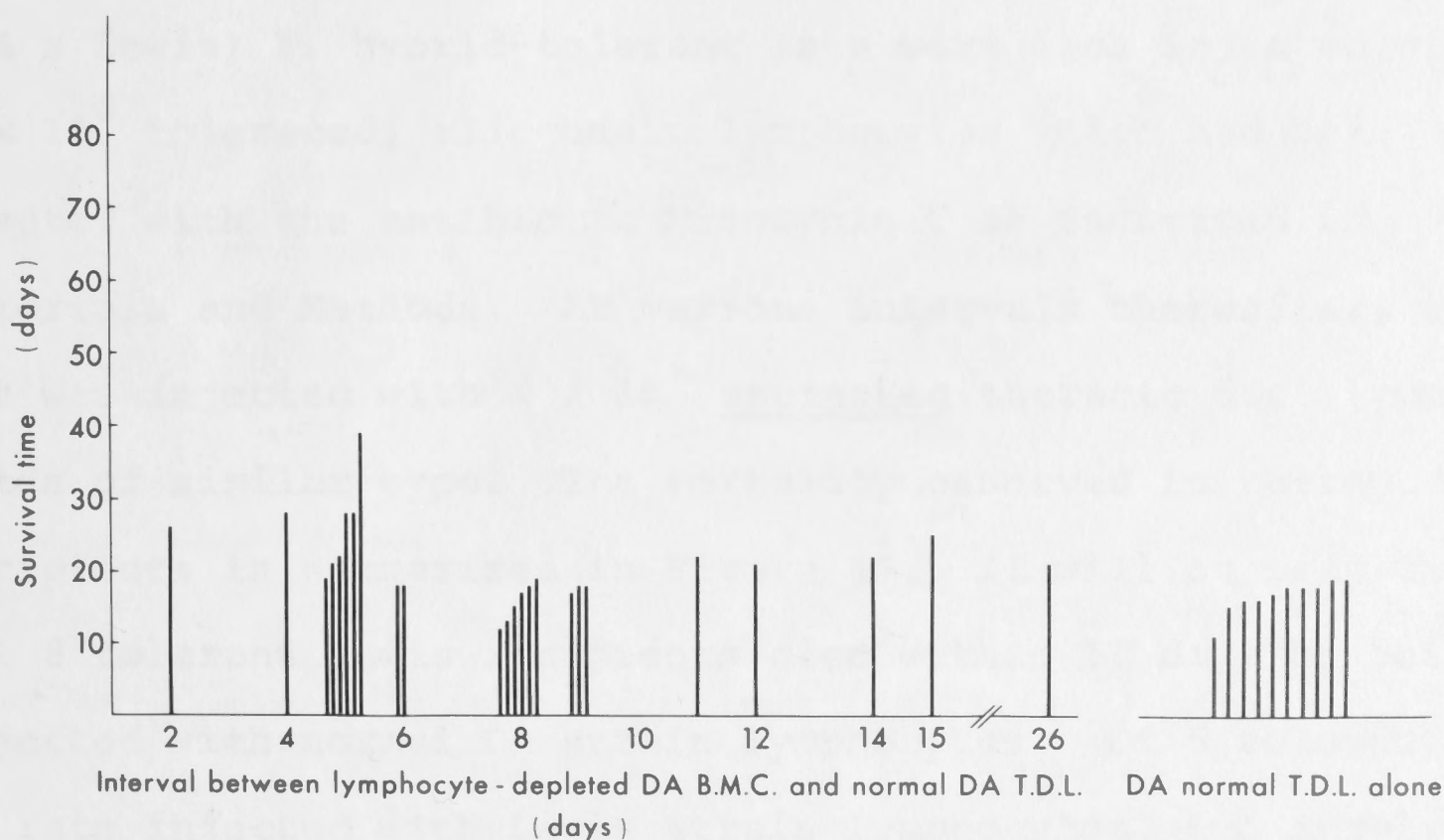
The effect of this procedure of administering an inoculum of bone marrow cells as a preliminary to the injection of allogeneic lymphocytes are summarized in Figures 9(a) and 9(b). (For comparison, the survival periods of tolerant rats injected with allogeneic lymphocytes alone, as previously described, have been included). There are two striking features of these figures. The first is the prolongation of life achieved in tolerant DA recipients of 4×10^8 Lewis strain lymphocytes as a result of the preliminary administration of bone marrow cells from Lewis donors. In this experiment, 15 out of 25 recipients remained alive more than 200 days after receiving a dose of lymphocytes which proved lethal within 16 days in all nine of the control rats (Figure 9(a)). The second feature of these results is that the identical preliminary procedure of injection of allogeneic (DA) bone marrow cells proved to be singularly fruitless in protecting tolerant Lewis rats from a subsequent injection of DA lymphocytes. Of 23 tolerant Lewis rats examined, the longest survivor died after 39 days and none of the remainder survived beyond 28 days (Figure 9(b)).

Apart from providing a source of haemopoietic stem

Fig. 9. Effect of pretreatment with lymphocyte-depleted bone marrow cells from tolerated, allogeneic donors.



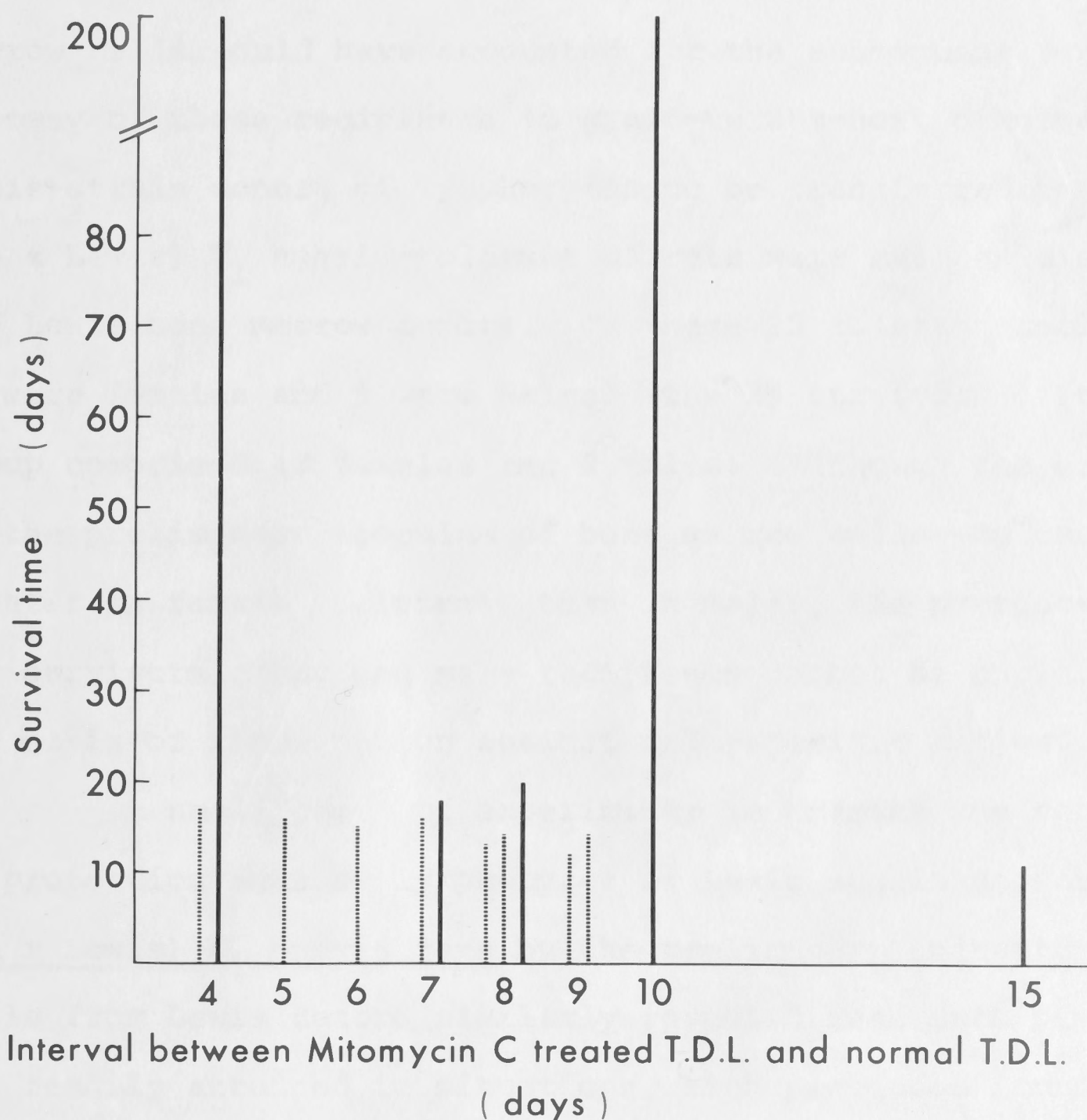
- (a) 2×10^8 Lewis strain bone marrow cells were injected into DA rats tolerant of (DA x Lewis) F_1 tissues. At the indicated intervals, 4×10^8 thoracic duct lymphocytes from Lewis rats were injected into the pretreated DA rats tolerant of (DA x Lewis) F_1 tissues.



- (b) 2×10^8 strain bone marrow cells were injected into Lewis rats tolerant of (DA x Lewis) F_1 tissues. At the indicated intervals, 4×10^8 thoracic duct lymphocytes from DA rats were injected into the pretreated Lewis rats tolerant of (DA x Lewis) F_1 tissues.

cells which would not be susceptible to attack by the allogeneic lymphocytes subsequently injected, the preliminary inoculum of bone marrow cells may have acted in other ways to mitigate graft-versus-host reactions. For instance, it is possible that an inoculum of lymphocyte-depleted, allogeneic bone marrow cells may have rendered the recipients incapable of responding with a graft-versus-host reaction when challenged with allogeneic lymphocytes in a manner analogous to that observed in F_1 hybrid rats which have been exposed to Mitomycin C-treated allogeneic lymphocytes (McCullagh, 1973). Alternatively, as thoracic duct lymphocyte donors were normally male rats, it is necessary to consider the possibility that preliminary challenge with bone marrow cells was inducing immunity against a male-specific antigen. Accordingly, some additional experiments were performed. In one of these, 13 (DA x Lewis) F_1 hybrid-tolerant rats were each injected with 4×10^8 tolerated, allogeneic lymphocytes which had been treated with the antibiotic Mitomycin C as indicated in Materials and Methods. At various intervals thereafter, each rat was injected with 4×10^8 untreated thoracic duct lymphocytes of similar type. The mortality observed in these recipients is summarized in Figure 10. It will be seen that all 8 tolerant Lewis recipients died within 17 days of being injected with normal DA strain lymphocytes. Of 5 tolerant DA rats injected with Lewis strain lymphocytes, two survived indefinitely. The latter result implies that some protection can be afforded against subsequent graft-versus-host disease by a preliminary inoculum which does not provide haemopoietic

Fig. 10. Effect of pretreatment with Mitomycin C treated thoracic duct lymphocytes from tolerated, allogeneic donors.



————— (DA x Lewis) F₁-tolerant DA rats were injected with 4×10^8 Mitomycin C treated Lewis strain thoracic duct lymphocytes, followed by 4×10^8 normal Lewis thoracic duct lymphocytes.

..... (DA x Lewis) F₁-tolerant Lewis rats were injected with 4×10^8 Mitomycin C treated DA thoracic duct lymphocytes, followed by 4×10^8 normal DA thoracic duct lymphocytes.

stem cells.

The experimental records were examined to determine whether immunization of the tolerant rats against male-specific antigens carried on the preliminary inoculum of bone marrow cells could have accounted for the subsequent resistance of many of these recipients to graft-versus-host disease. All Lewis-strain donors of lymphocytes to be transferred to (DA \times Lewis) F_1 hybrid-tolerant DA rats were male as also were the Lewis bone marrow donors. Of these 25 tolerant recipients, 16 were females and 9 were males. The 15 survivors of this group comprised 13 females and 2 males. Although the efficacy of the preliminary inoculum of bone marrow cells was much greater in female recipients than in males, the presence of any survivors among the male recipients cannot be explained on the basis of immunization against male-specific antigen.

A small group of experiments to examine the conferring of protection against lymphocytes of Lewis strain on normal (DA \times Lewis) F_1 hybrid rats by the preliminary injection of cells from Lewis donors similarly revealed that such protection was readily attained in situations which precluded immunization against male-specific antigens. Thus, 2 out of 3 female F_1 hybrid recipients of bone marrow cells from female Lewis rats subsequently survived challenges with large doses of lymphocytes from male Lewis donors. Similarly, of 7 male F_1 hybrids previously exposed to lymphocyte-depleted inocula of spleen cells from male Lewis rats, all 3 challenged with lymphocytes from male Lewis donors and 2 out of 4 challenged with lymphocytes from female donors survived indefinitely.

A final observation which, although it concerns a small number of rats, bears on the protective mechanism of preliminary inocula of bone marrow cells in tolerant rats was made in DA rats tolerant of Lewis (as distinct from (DA x Lewis) F₁ hybrid) tissues. Such rats, like animals tolerant of F₁ hybrid tissues, uniformly succumb after the transfer of 4×10^8 thoracic duct lymphocytes from Lewis rats. However, when given a preliminary inoculum of bone marrow cells from Lewis rats, two out of three Lewis-tolerant DA rats survived indefinitely.

3) Modification of graft-versus-host disease by subsequent administration of syngeneic lymphocytes

The evolution of graft-versus-host disease in neonatal rats injected with allogeneic lymph node cells can be aborted by the administration of lymphocytes from donors syngeneic with the neonates and sensitized against the allogeneic cell donors (Silvers and Billingham, 1969b). The capacity of thoracic duct lymphocytes from normal (non-sensitized) donors syngeneic with tolerant rats to protect those rats from the lethal effects of tolerated allogeneic lymphocytes was examined in the course of the present experiments. Complete reversal could be achieved provided that the syngeneic cells were administered sufficiently early. The results of these experiments are presented in the succeeding section together with observations on the fate of skin grafts on these rats.

F. The fate of skin grafts on homograft-tolerant rats following the transfer of tolerated, allogeneic lymphocytes

As has been previously reported (Billingham and

Silvers, 1961b; Martinez, Smith and Good, 1961) and was confirmed in the preceding section, the injection of a homograft-tolerant animal with lymphoid cells from a donor of the tolerated strain can produce a lethal graft-versus-host reaction. However, the lethal effects of an inoculum of Lewis strain lymphocytes are frequently not observed if (DA x Lewis) F_1 hybrid-tolerant DA rats have been injected previously with bone marrow cells from Lewis donors.

The present section reports observations made on skin grafts borne by tolerant rats following the transfer of tolerated, allogeneic lymphocytes. In one group, the tolerant recipients succumbed to a graft-versus-host reaction, while the rats in the other group were protected by means of the preliminary injection of bone marrow cells from donors of similar strain to the lymphocyte donors. In an attempt to delineate the influences to which these grafts were being subjected, the course of parental-strain skin grafts placed on normal (DA x Lewis) F_1 hybrids was followed after the injection of lymphocytes from donors of the opposite parental strain. Furthermore the relative susceptibility of long-standing and recently placed skin grafts to these procedures was compared.

1) The response of skin grafts on tolerant rats to the transfer of lymphocytes, in the absence of other treatment

Grafts of (DA x Lewis) F_1 hybrid skin which had been placed on 19 (DA x Lewis) F_1 hybrid tolerant parental strain rats more than 50 days previously remained macroscopically intact at the time of death of the recipients, 11 to 19 days

after the receipt of 4×10^8 tolerated allogeneic thoracic duct lymphocytes. Histological examination of these grafts failed to reveal any abnormality other than a slight dermal mononuclear infiltration (Figs 11, 15). This survival of skin grafts for 11 to 19 days after allogeneic lymphocyte transfer contrasts with the rejection of similar grafts by tolerant rats 12 to 16 days after the transfer of 4×10^8 syngeneic lymphocytes.

It is possible that long-standing skin grafts may be less sensitive indicators of anti-graft activity than grafts which are still in the process of healing in. To avoid this disadvantage, the course of grafts which were placed either 7 days previously, or at the time of lymphocyte injection, was studied. Additionally, as skin grafts from F_1 hybrid donors are likely to be susceptible to attack from both the injected lymphocytes and from any host cells which have regained reactivity, grafts syngeneic with the transferred lymphocytes were also placed on these tolerant rats. (DA x Lewis) F_1 hybrid-tolerant Lewis rats were grafted with skin from F_1 hybrid donors shortly before receiving 4×10^8 normal DA strain lymphocytes. Two rats received an additional graft of DA strain skin while the other two were also grafted with skin from a Lewis donor. At the time of death of these four rats (15-17 days after lymphocyte injection) both long-standing and freshly placed F_1 hybrid skin grafts appeared to be intact macroscopically. Histological examination of the F_1 hybrid strain skin revealed some foci of epidermal necrosis together with diffuse mononuclear infiltration, these changes being more

noticeable in the recently placed grafts (Figure 12). When the grafts of DA and Lewis strain skin were examined microscopically, the changes observed in the freshly placed F_1 hybrid grafts were duplicated in the Lewis skin (Figure 14) whereas the grafts of DA skin (Figure 13) were free from necrosis and infiltration.

The converse situation of that examined in the preceding experiments, namely the survival of skin grafts on DA rats tolerant of (DA x Lewis) F_1 hybrid tissues following transfer of Lewis strain lymphocytes, was also examined. Four tolerant DA rats were freshly grafted with both F_1 hybrid and Lewis strain skin shortly before the injection of 4×10^8 lymphocytes from Lewis rats. At the time of death (14 to 16 days after lymphocyte transfer) both of the freshly placed grafts (F_1 hybrid and Lewis) and the long-standing F_1 hybrid graft appeared to be in good condition on all rats. Histological examination confirmed this assessment, there being very few indications of necrosis or cellular infiltration in any of the grafts (Figs 15, 16).

2) The response of skin grafts on normal F_1 hybrid rats to the transfer of lymphocytes from parental strain donors

It was found in Section 4F, 1 that grafts of F_1 hybrid skin placed on F_1 hybrid-tolerant rats of one parental strain remained in remarkably good condition at the time of death from graft-versus-host disease following the injection of lymphocytes from donors of the other parental strain. This observation was of interest in view of the complete, and macroscopically evident rejection of grafts of F_1 hybrid skin

reported (in Section 4,A) to occur within this time following the transfer of normal syngeneic lymphocytes to F_1 hybrid-tolerant hosts. In order to explain the improved survival of F_1 hybrid skin grafts in tolerant rats dying of graft-versus-host disease, a number of possibilities were considered. For example, the disordered pathophysiology of a rat succumbing to this disease may be unfavourable to the mounting of a homograft response. Alternatively, it is possible that the occurrence of a state in which graft survival is actively encouraged in tolerant rats (as implied by the previous experiments in which passage of cells capable of abrogating tolerance was undertaken - Section 4,B) might be responsible. The examination of skin graft survival on F_1 hybrid rats succumbing to graft-versus-host disease provided a means of testing for any depressing effect of this state on homograft rejection mediated by transferred lymphocytes. Furthermore, it could facilitate evaluation of the role of any active enhancement of graft survival in tolerant rats.

Six normal (DA x Lewis) F_1 hybrid rats received grafts of skin from both F_1 hybrid and Lewis rats and, shortly afterwards, were each injected intravenously with 4×10^8 lymphocytes from normal DA rats. All rats died, with the typical features of graft-versus-host disease, between 14 and 16 days after injection. At the time of death, both grafts appeared, macroscopically, to be necrotic in five rats. In the sixth F_1 hybrid, the grafts were in better condition macroscopically. Histological examination confirmed these impressions. In both grafts on the first five rats, there was

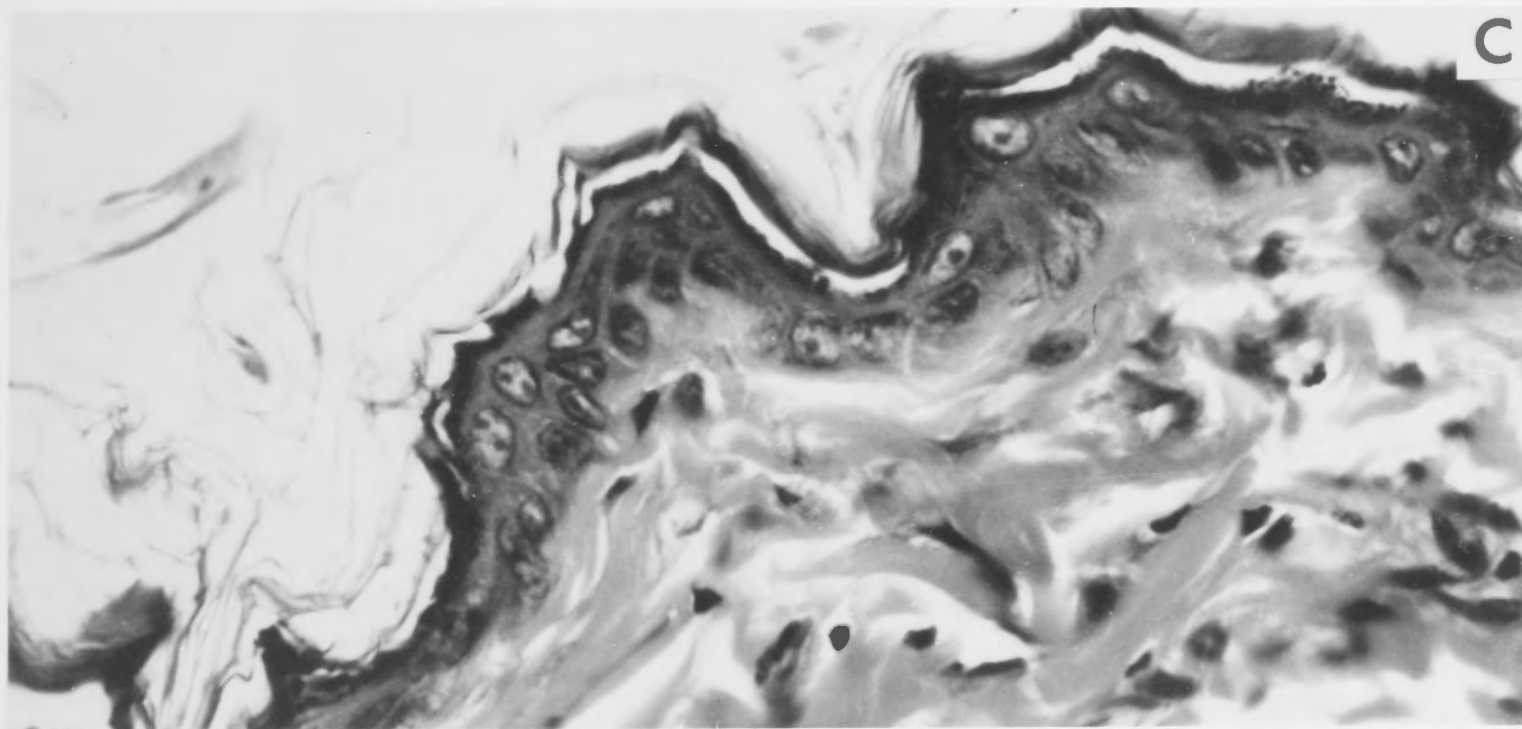
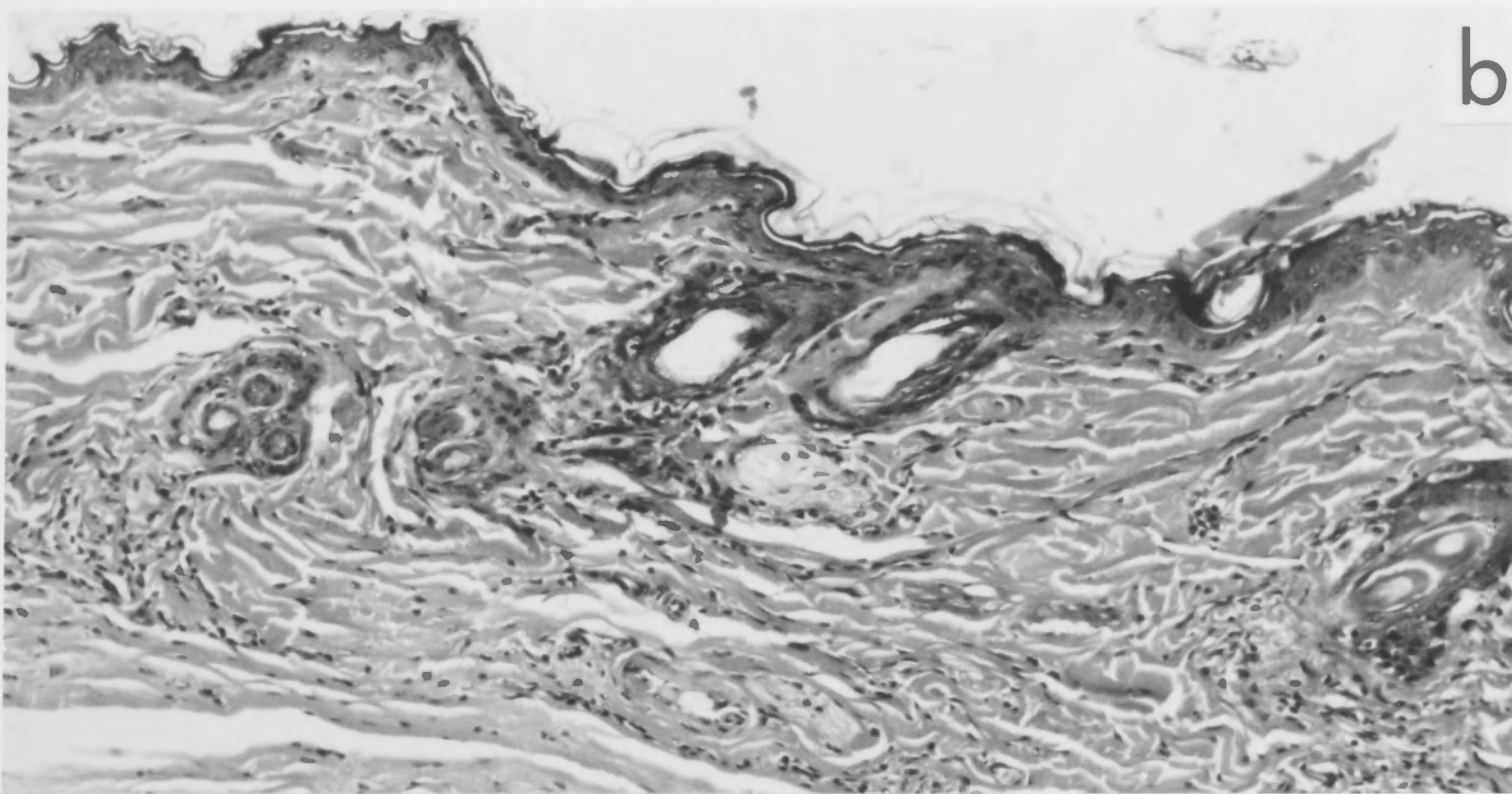
diffuse necrosis of the epidermis accompanied by a heavy polymorphonuclear infiltration and the complete destruction of all epidermal appendages (Figs 17, 18). The grafts on the sixth F_1 hybrid, although exhibiting considerable necrosis of the epidermis and its appendages, were in appreciably better condition being reminiscent of the grafts borne by tolerant rats examined in Section 4,F.1.

Comparison of skin grafts on tolerant parental strain and F_1 hybrid rats suffering from graft-versus-host disease does not support the contention that the relative preservation of the former at the time of death is attributable to the concomitant graft-versus-host disease. However, the early stage at which tolerant parental strain rats succumbed precluded any assessment of whether prolonged survival of their skin grafts was possible. To elucidate this point, recourse was had to the technique previously described (Section 4,E) for preventing the occurrence of graft-versus-host disease in tolerant rats in receipt of tolerated allogeneic lymphocytes.

3) The response of skin grafts on tolerant rats, which have been protected against graft-versus-host disease, following the transfer of normal lymphocytes

Ten DA rats tolerant of (DA x Lewis) F_1 hybrid tissues were each injected with 2×10^8 bone marrow cells obtained from Lewis rats which had been depleted of lymphocytes by prolonged drainage from a thoracic duct fistula. From 8 to 10 days thereafter, these rats received a fresh graft of (DA x Lewis) F_1 hybrid skin (in addition to the long-standing graft of this skin which had served to confirm their tolerant state) and were

injected with 4×10^8 thoracic duct lymphocytes from Lewis donors. All ten rats survived this inoculum of lymphocytes (which was uniformly lethal in the absence of pre-treatment with bone marrow cells). The course followed by their skin grafts is summarized in Table 15. In the case of six rats, both freshly placed and long-standing grafts remained healthy without any suggestion of a homograft response. In a further two tolerant DA recipients, the freshly placed F_1 hybrid skin grafts became erythematous during the second and third weeks. In the fourth, fifth and sixth weeks after the injection of Lewis-strain lymphocytes, both old and new grafts were subject to extensive depilation in both animals. However, during the latter part of the second month, complete regrowth of fur occurred and all four grafts regained (and retained) a completely healthy appearance. In the two remaining rats, the long-standing skin grafts remained intact after lymphocyte injection. However, in both cases, the freshly placed grafts passed through successive phases of erythema and desquamation to assume a completely necrotic appearance. By the fifth week, the fresh grafts on both rats were reduced to eschars macroscopically indistinguishable from those produced in homograft rejection by normal hosts. However, the further course of these grafts was quite surprising in that both eschars sloughed to reveal healthy F_1 hybrid skin. The subsequent regrowth of fur on these grafts restored them to a completely normal appearance by the end of the second month. These stages are shown in Figure 19.



In figs. 11-18, photomicrograph (a) is at 45 x magnification, (b) at 180 x magnification and (c) at 720 x magnification.

Fig. 11. Long-tolerated (DA x Lewis) F_1 hybrid skin graft on Lewis rat tolerant of (DA x Lewis) F_1 hybrid tissues following the injection of 4×10^8 T.D.L. from a normal DA rat. Skin taken at the time of death as a result of G.v.H. disease.

Note that the epidermis and sebaceous gland are intact and there is a slight dermal mononuclear infiltration.

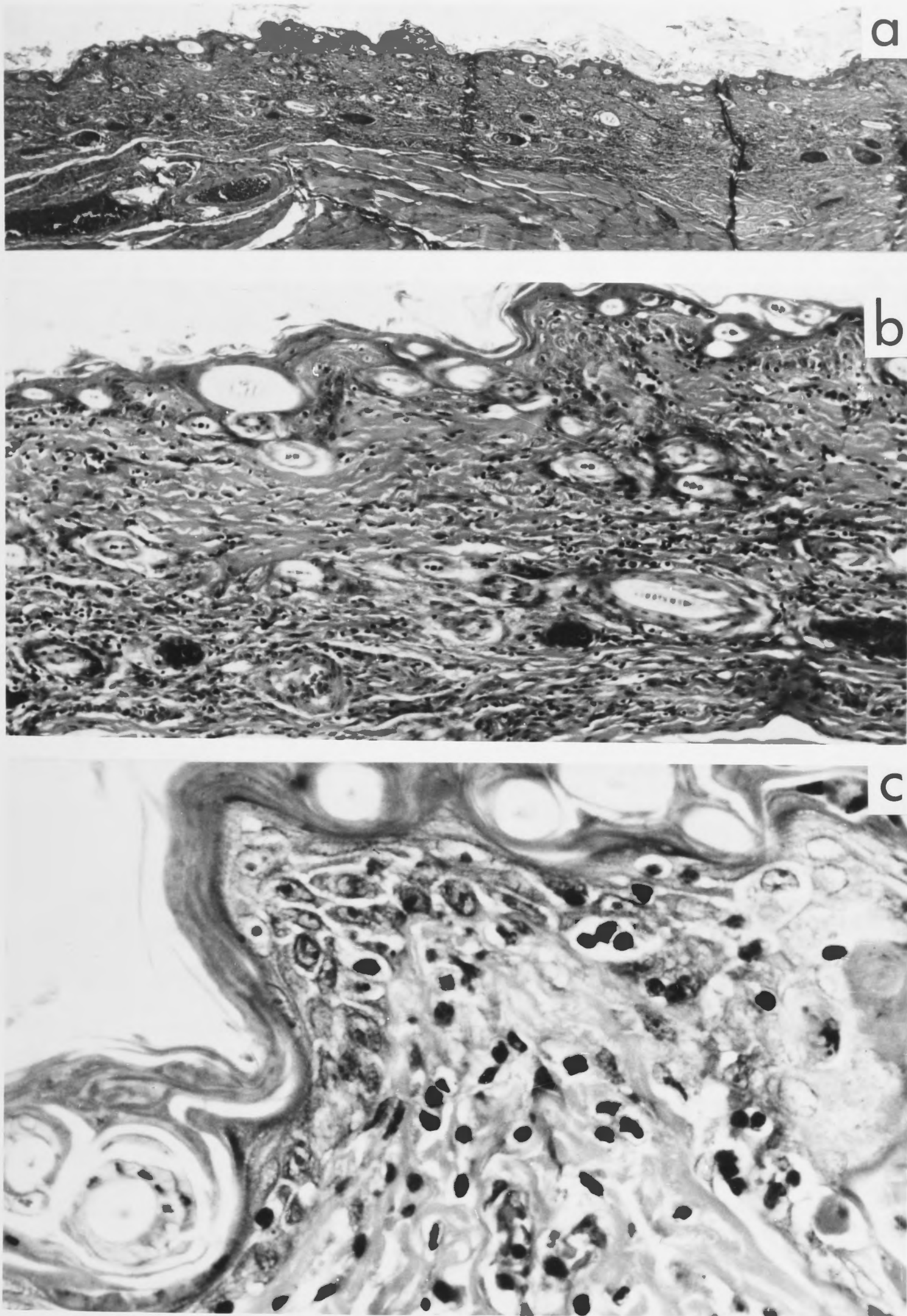


Fig. 12. (DA x Lewis) F_1 hybrid skin graft freshly placed on a Lewis rat tolerant of (DA x Lewis) F_1 hybrid tissues which has been injected with 4×10^8 T.D.L. from a normal DA rat. Specimen obtained at the time of death as a result of G.v.H. disease.

Note that there are some foci of epidermal necrosis together with a diffuse dermal mononuclear infiltration.

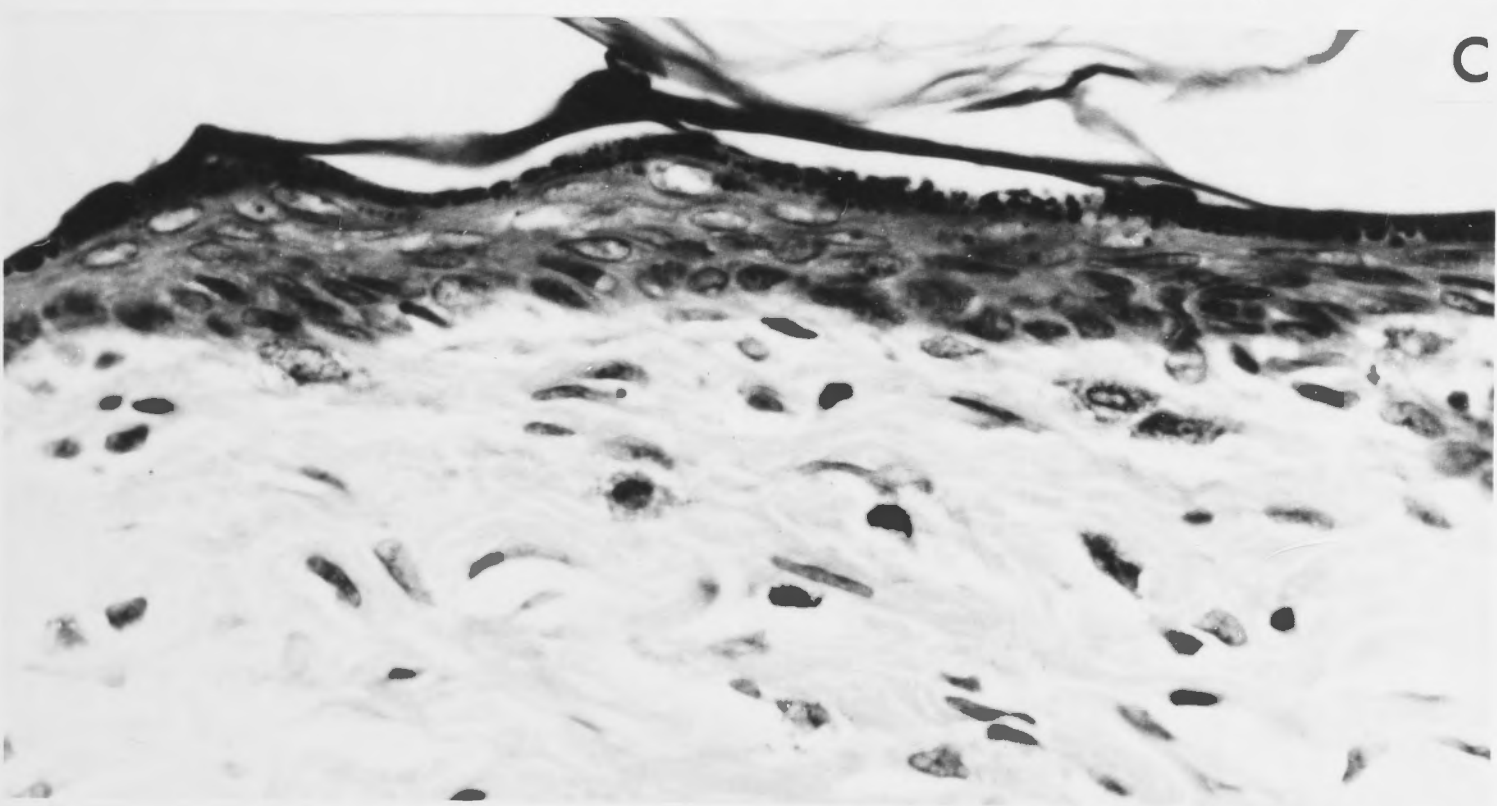
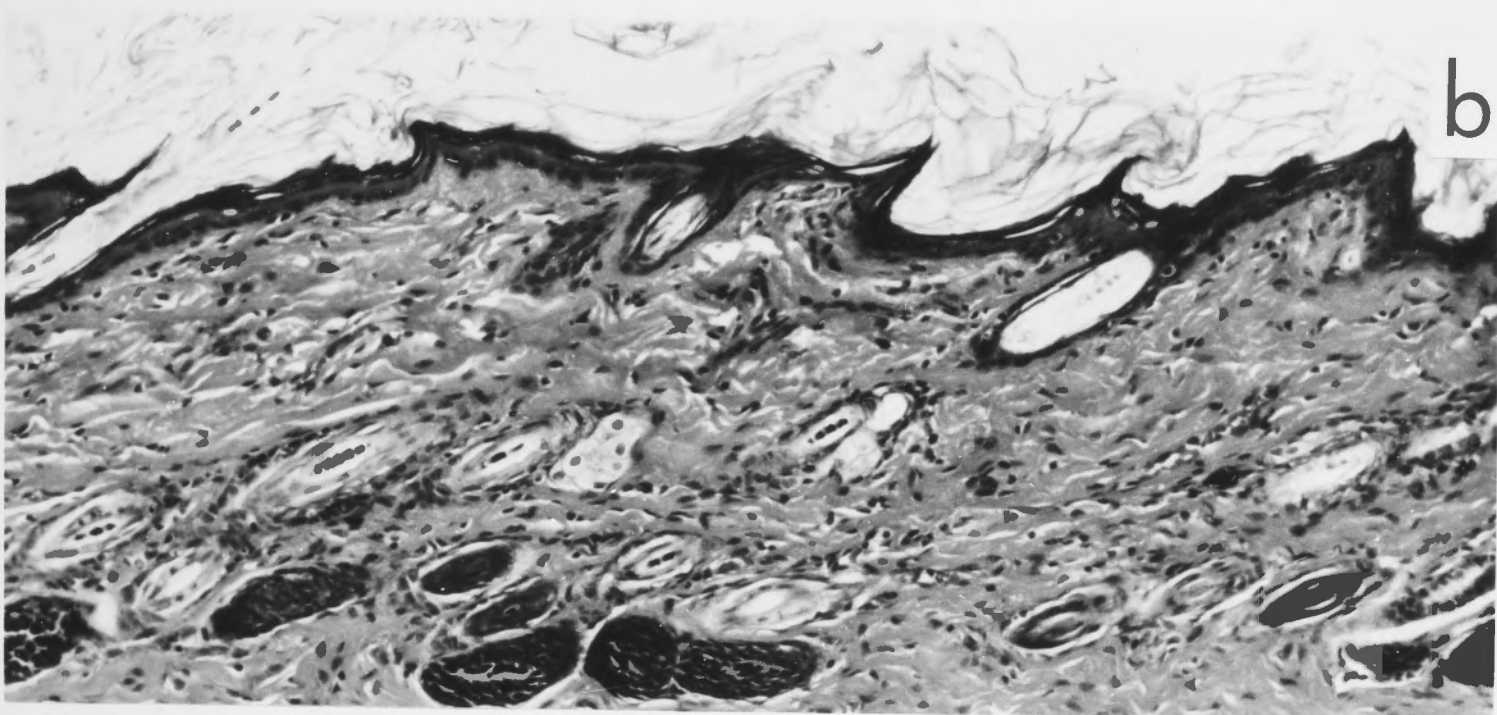
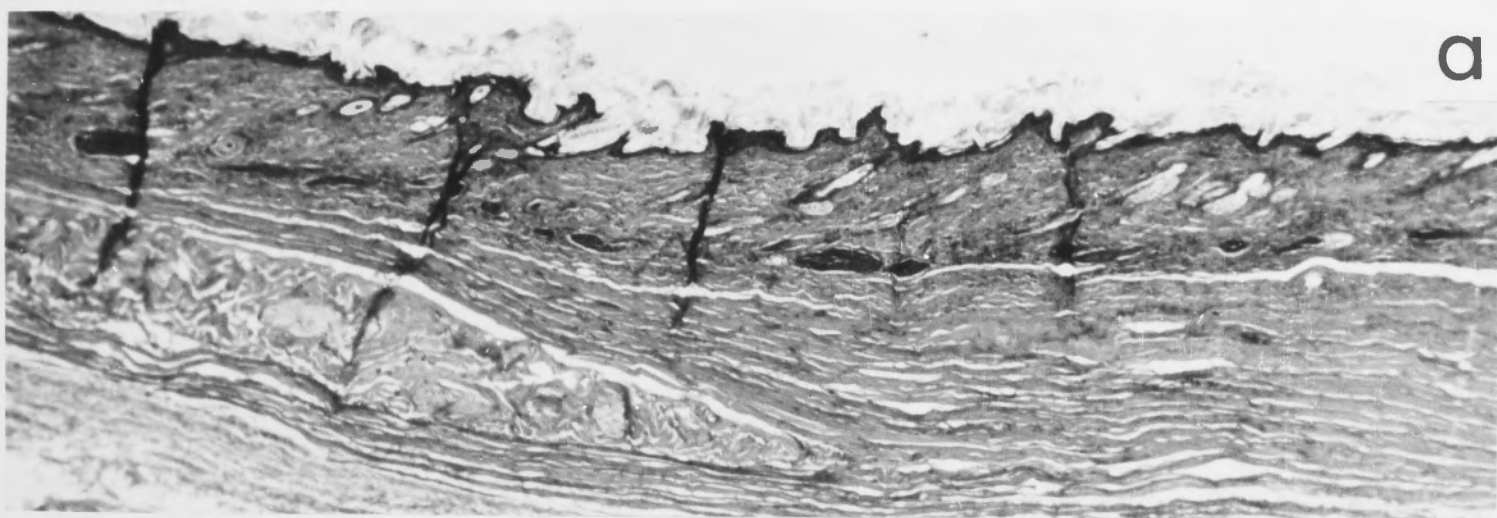


Fig. 13. DA skin graft freshly placed on a Lewis rat tolerant of (DA x Lewis) F_1 hybrid tissues which has been injected with 4×10^8 T.D.L. from a normal DA rat examined at the time of death as a result of G.v.H. disease.

Note the absence of any signs of rejection reaction.

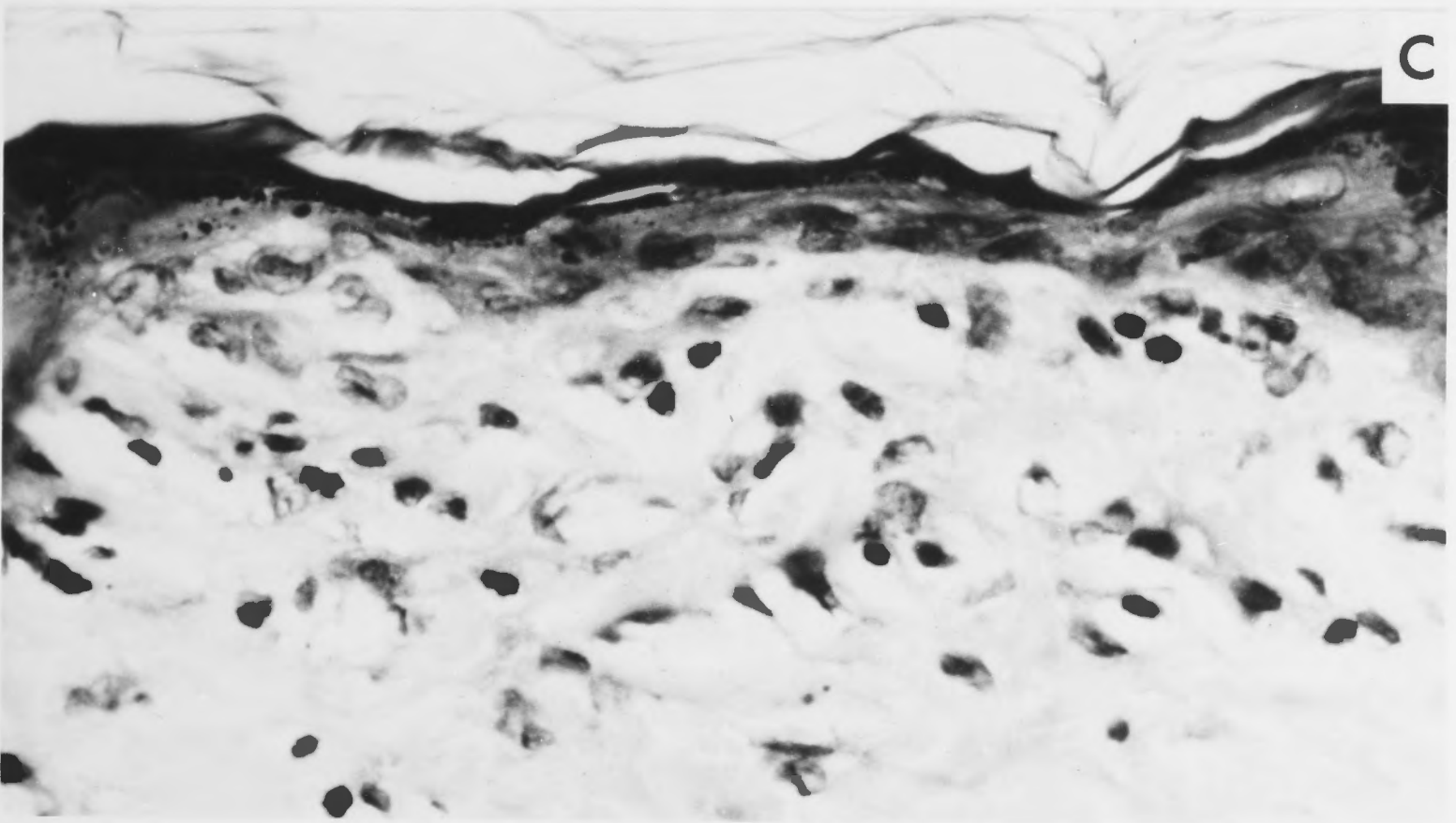
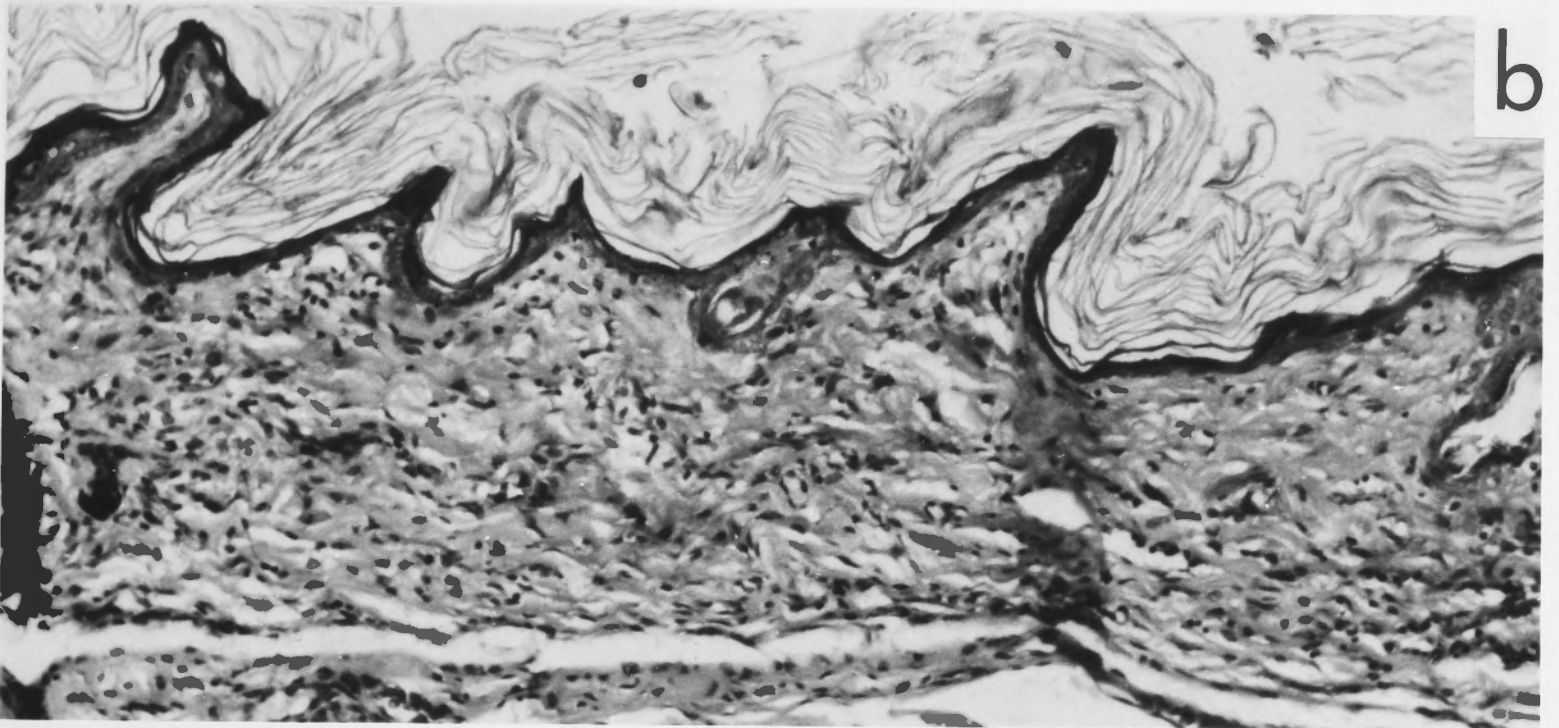
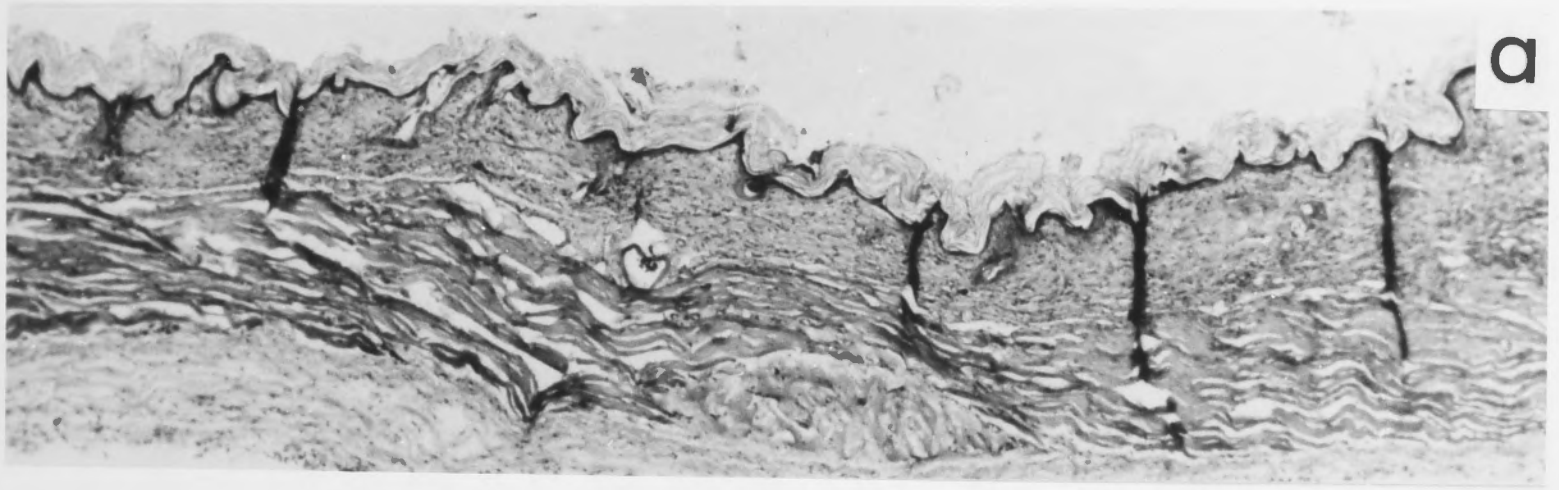


Fig. 14. Freshly placed Lewis skin graft on a Lewis rat tolerant of (DA x Lewis) F_1 hybrid tissues which has been injected with 4×10^8 T.D.L. from a normal DA rat examined at the time of death as a result of G.v.H. disease.

Note the presence of some foci of epidermal necrosis together with diffuse dermal mononuclear infiltration.

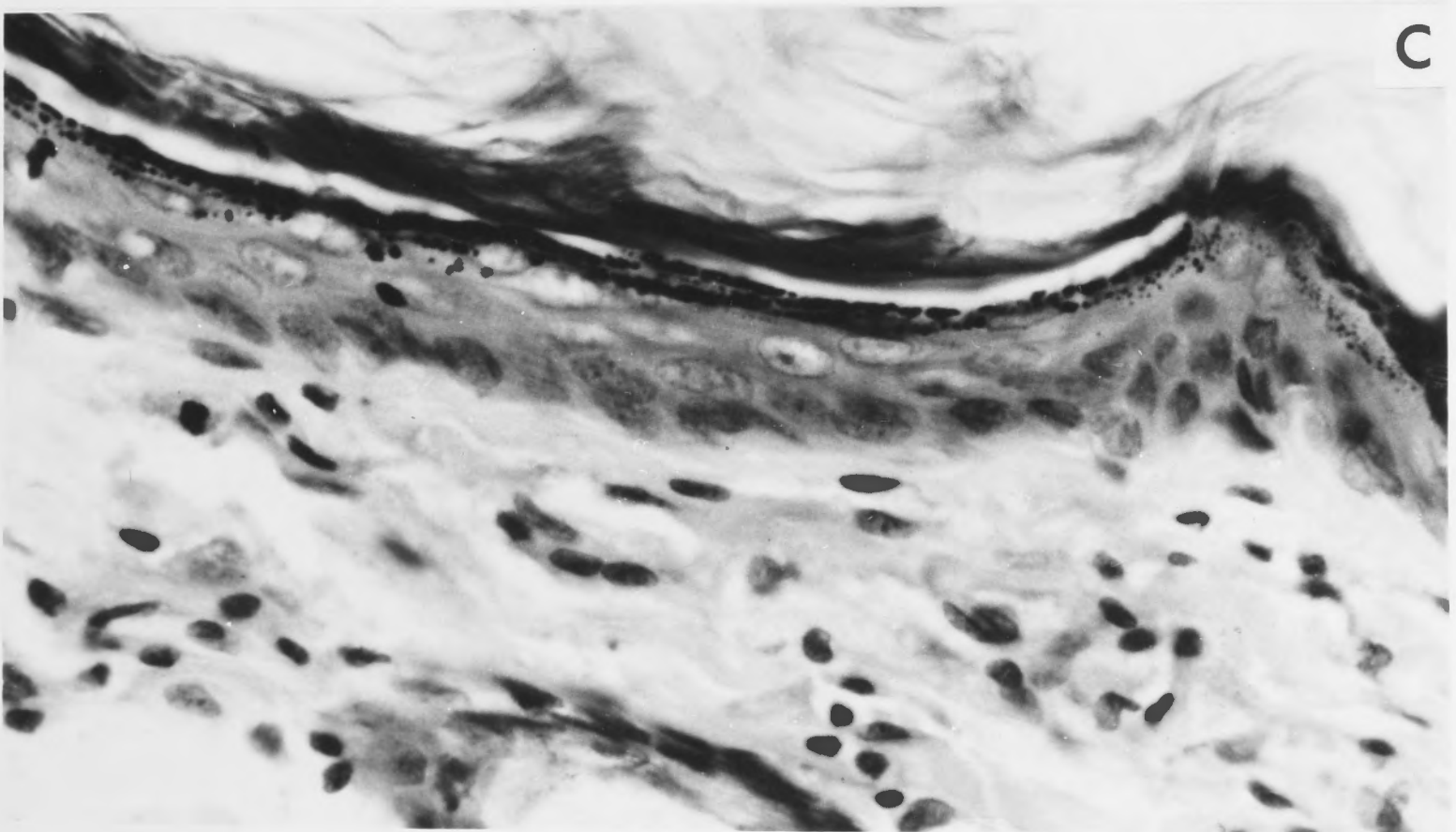
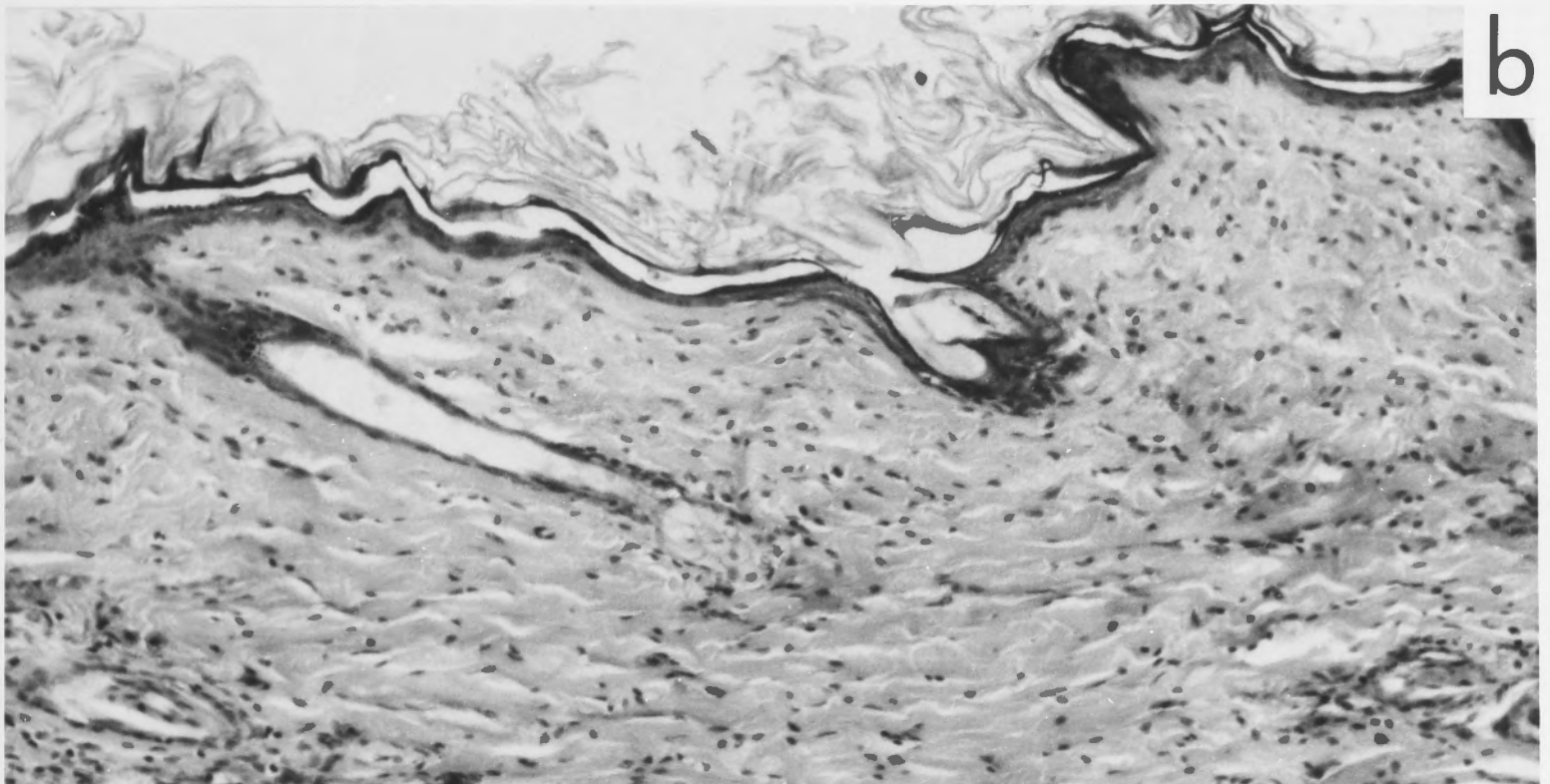
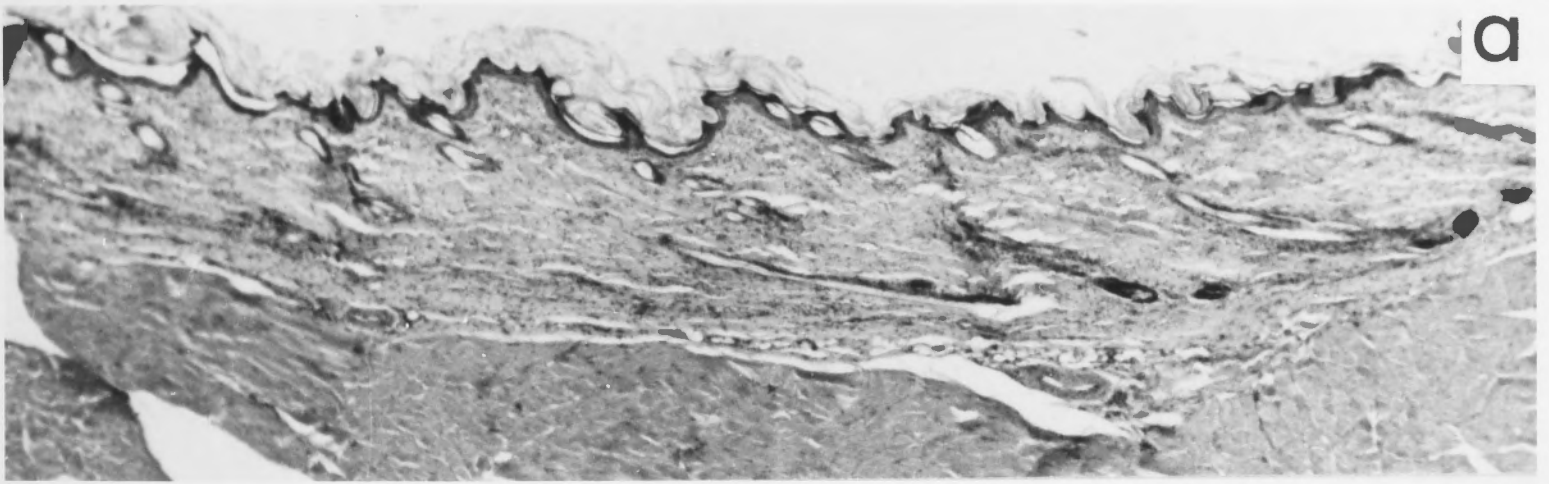


Fig. 15. Long-tolerated (DA x Lewis) F_1 hybrid skin graft on a DA rat tolerant of (DA x Lewis) F_1 hybrid tissues following the injection of 4×10^8 T.D.L. from a normal Lewis rat, at the time of death as a result of G.v.H. disease.

Note that epidermis and sebaceous gland are intact. A slight dermal mononuclear infiltration is observed.

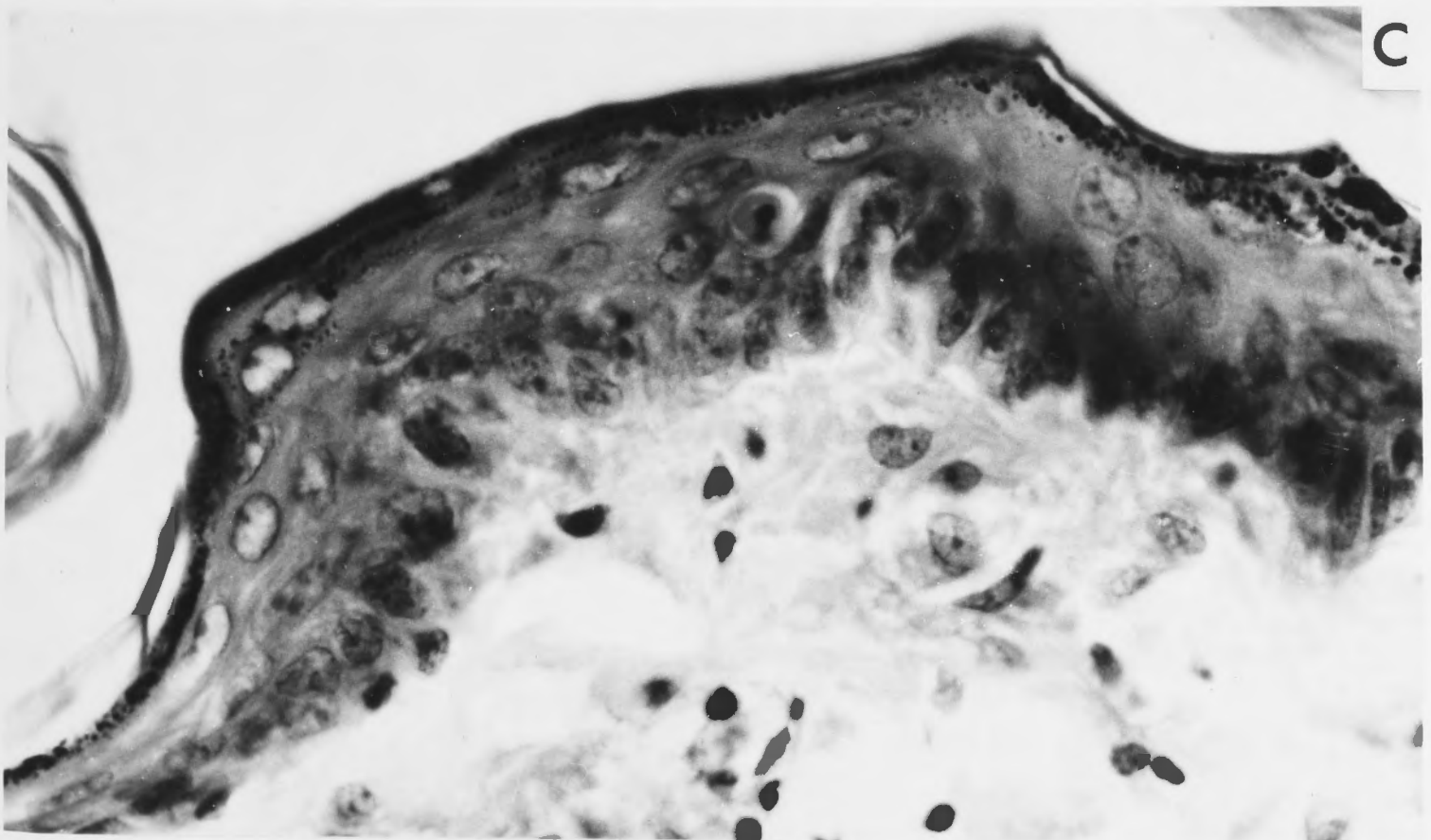
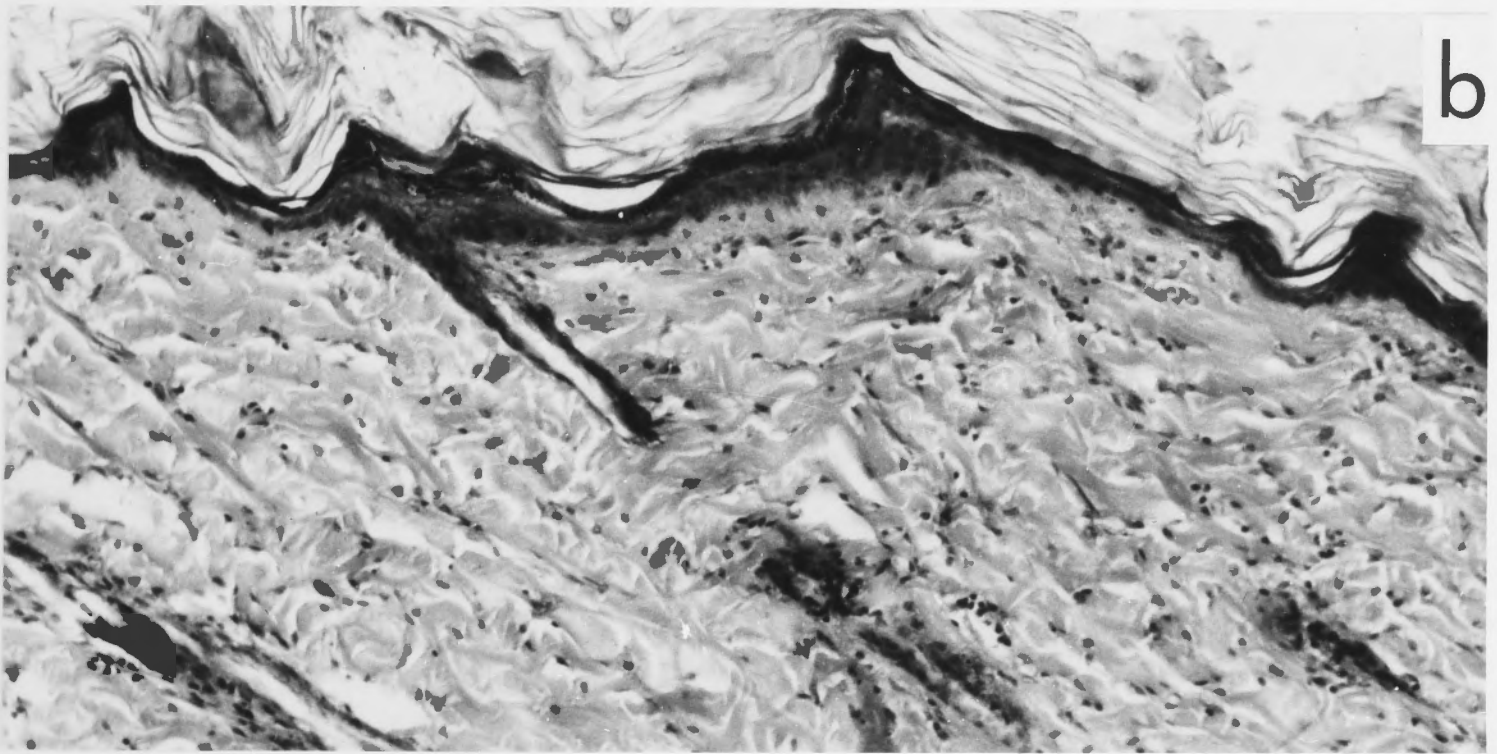
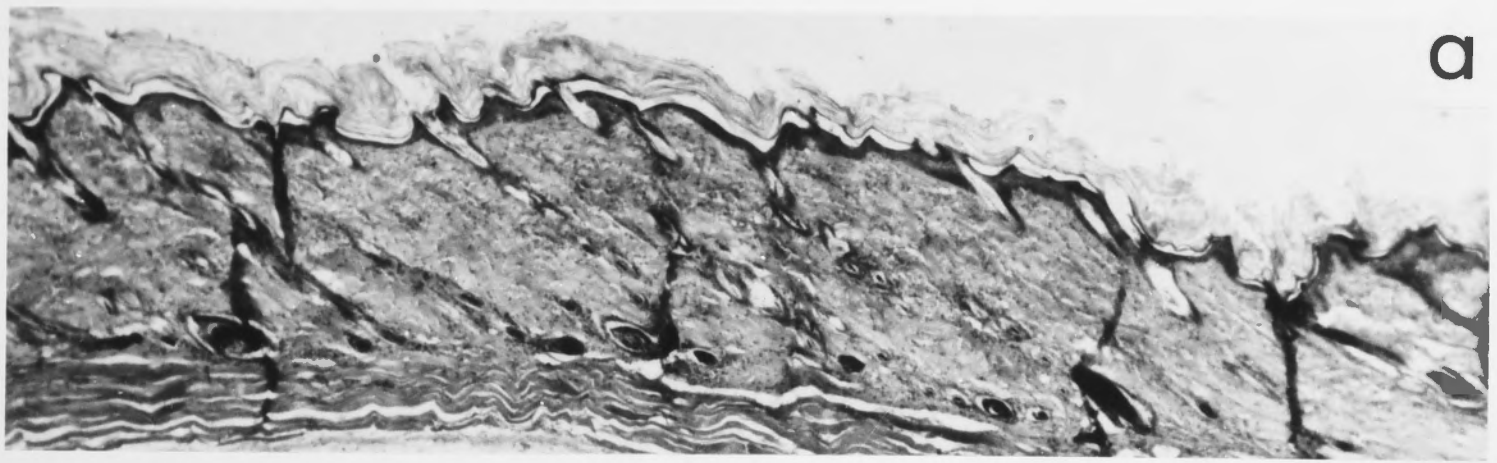


Fig. 16. Freshly placed (DA x Lewis) F_1 hybrid skin graft on a DA rat tolerant of (DA x Lewis) F_1 hybrid tissues following the injection of 4×10^8 T.D.L. from a normal Lewis rat. Specimen was taken at the time of death as a result of G.v.H. disease.

Note that the epidermis is completely intact and a slight dermal mononuclear infiltration is visible.

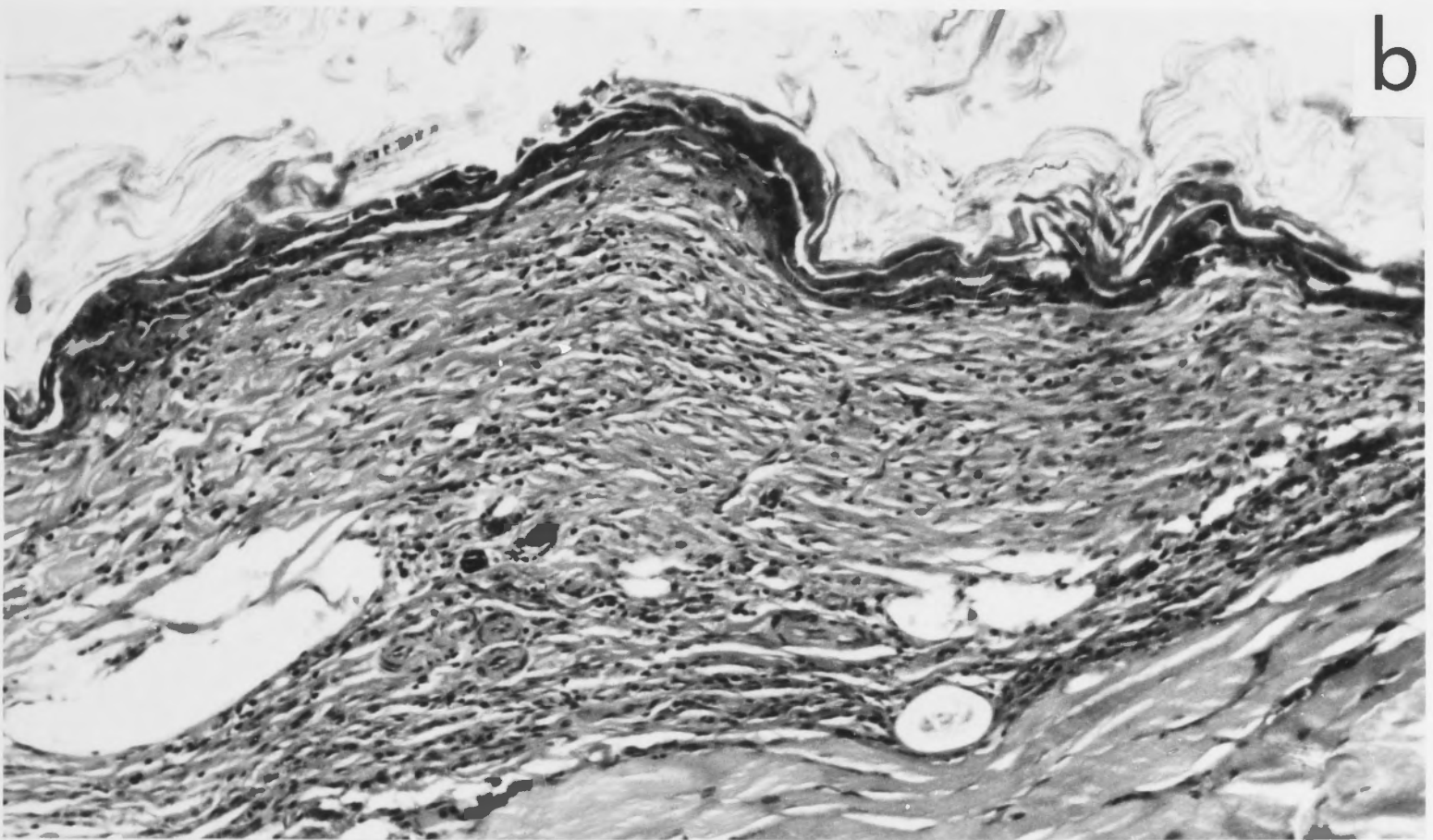
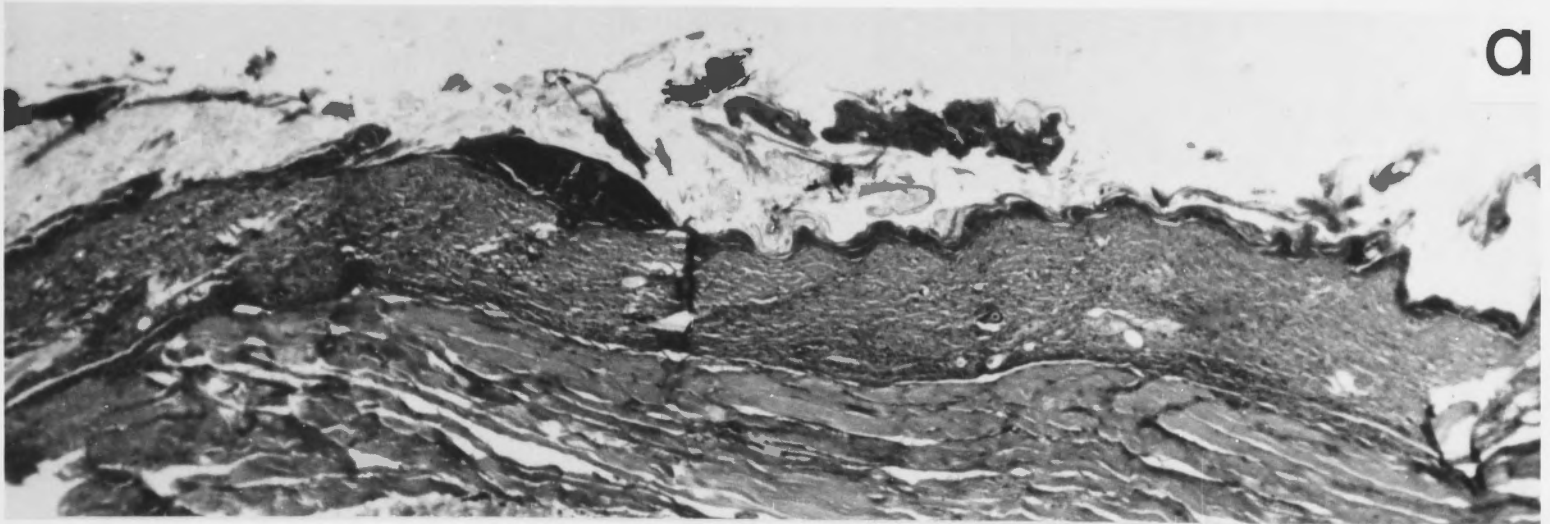


Fig. 17. Freshly placed (DA x Lewis) F_1 hybrid skin graft on a (DA x Lewis) F_1 hybrid rat injected with 4×10^8 T.D.L. from a normal DA rat examined at the time of death as a result of G.v.H. disease.

Note the diffuse necrosis of the epidermis and the diffuse mononuclear infiltration.

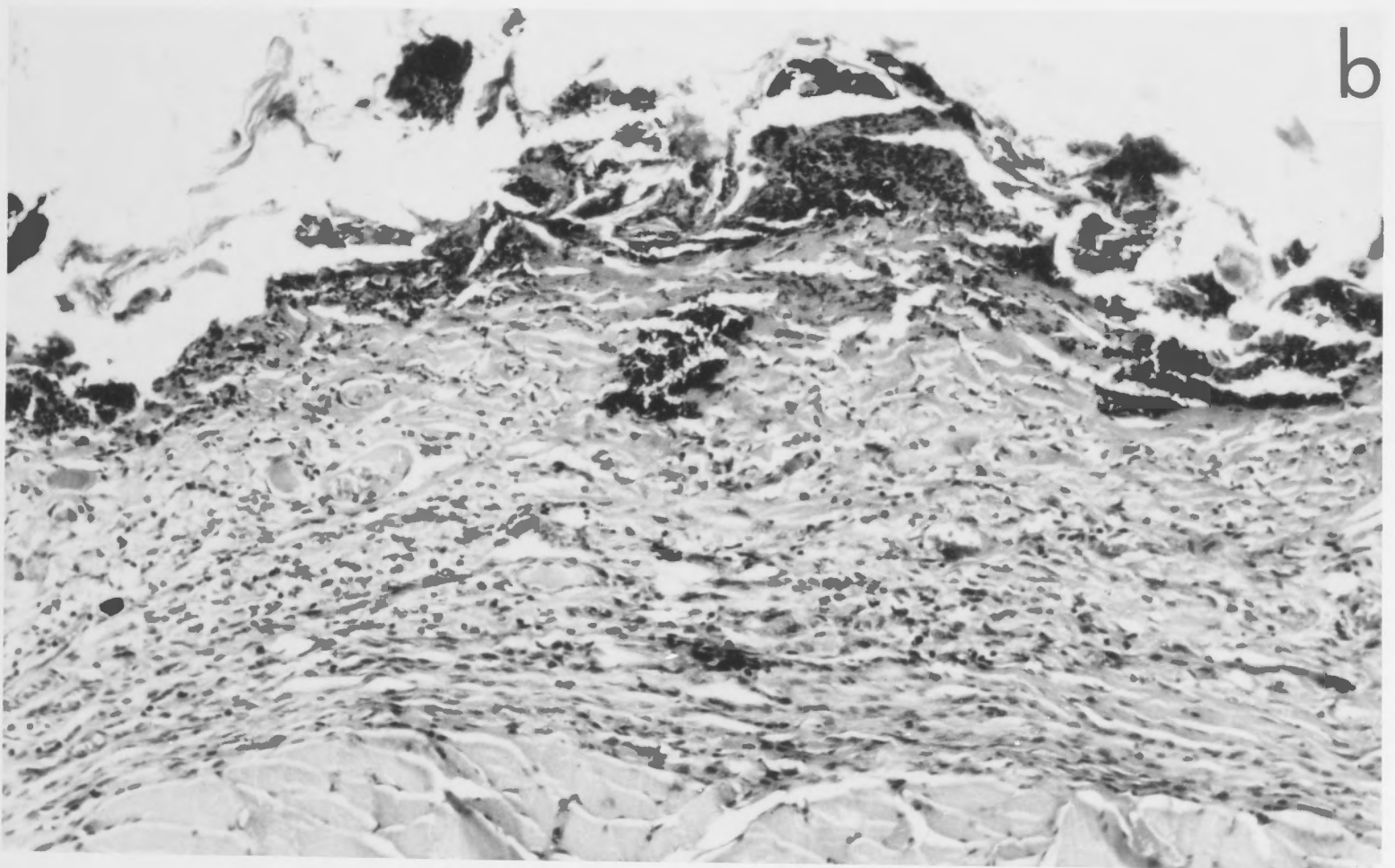


Fig. 18. Freshly placed Lewis skin graft on a (DA x Lewis) F_1 hybrid rat injected with 4×10^8 T.D.L. from a normal DA rat, taken at the time of death as a result of G.v.H. disease.

Note the diffuse necrosis of the epidermis together with a heavy polymorphonuclear infiltration.

TABLE 15

- * (DA x Lewis) F_1 -tolerant DA rats were injected with 2×10^8 "T.D.L. depleted" Lewis B.M.C., followed, 8-10 days later, by 4×10^8 normal Lewis T.D.L.
- † "depleted" B.M.C. were obtained from male Lewis rats which were drained of T.D.L. for 4-5 days.
- ‡ New (DA x Lewis) F_1 skin was placed on tolerant DA rats on the same day as the transfer of normal Lewis T.D.L.
- § Long-established (tolerated) (DA x Lewis) F_1 hybrid skin grafts.

TABLE 15

Rejection-recovery episodes of (DA x Lewis) F₁ hybrid skin grafts on tolerant DA rats*.

Tolerant Rat No.	Interval between B.M.C. & T.D.L.	Day after injection of normal Lewis T.D.L. and skin grafting of new (DA x Lewis) F ₁ hybrid skin [±] on tolerant DA rats pre-treated with T.D.L.-depleted B.M.C. [±] from Lewis donors									
		10	20	30	40	50					
1	8	● new F ₁ skin grafts became red → became slightly hard to touch → complete depilation → complete recovery									
2	8	● old F ₁ skin grafts; depilated along the border line between skin graft and host → complete recovery									
3	8) old skin grafts § remained intact during rejection-recovery episodes									
		12	20	30	37						
		● new F ₁ skin graft became red and shrank in size → completely necrotic appearance → hard scab → new fur growth again → complete recovery									
4	8) slight desquamation occurred on new F ₁ skin graft									
		20	26	30	46	50					
		● slight desquamation occurred on new F ₁ skin graft → Necrotic change started → Scab formation → hard scab shed → new fur growth again → complete recovery									
5	8	No rejection-recovery episodes →									
6	8										
7	9										
8	9										
9	9										
10	10										

Fig. 19. "Rejection-recovery" episodes of a (DA x Lewis) F_1 hybrid skin graft on a DA rat tolerant of (DA x Lewis) F_1 hybrid tissues. A tolerant DA rat bearing a long-tolerated (DA x Lewis) F_1 hybrid skin graft was injected with 2×10^8 lymphocyte-depleted Lewis bone marrow cells, followed, 8 days later, by 4×10^8 normal Lewis thoracic duct lymphocytes. A fresh (DA x Lewis) F_1 hybrid skin graft was placed on the same day as the transfer of normal Lewis thoracic duct lymphocytes (See tolerant rat No. 4 in Table 15).

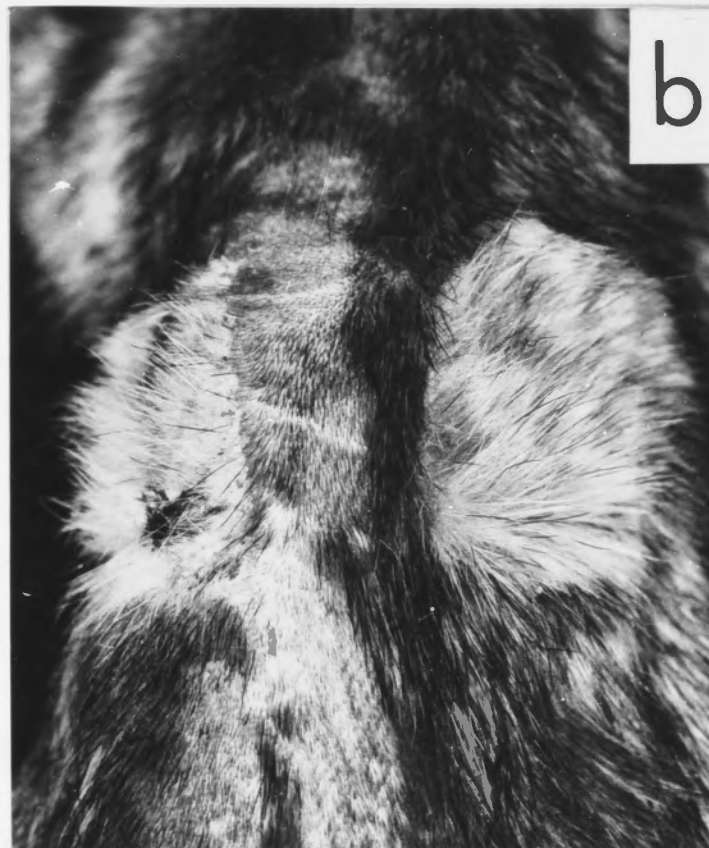
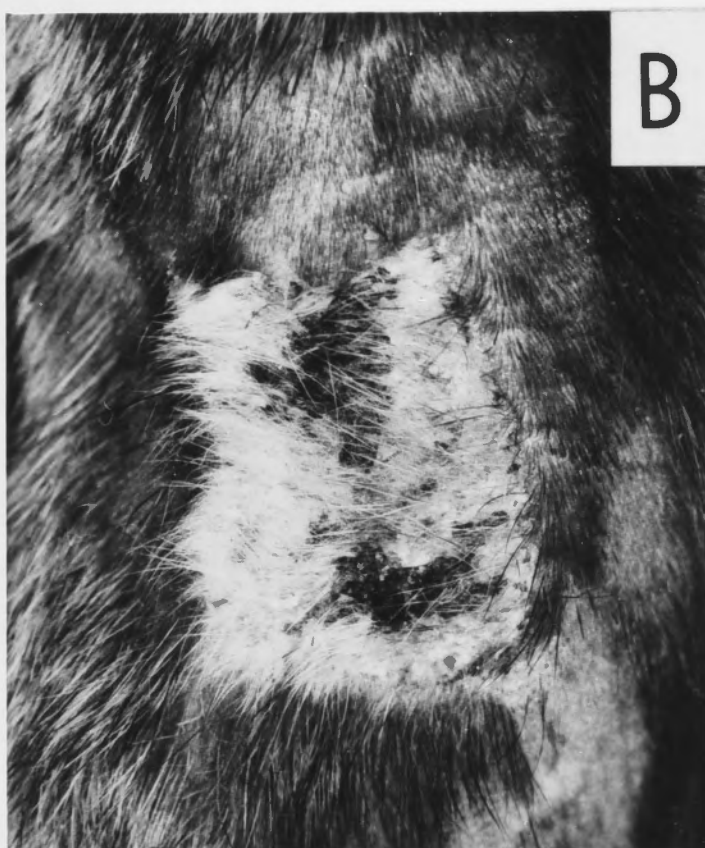
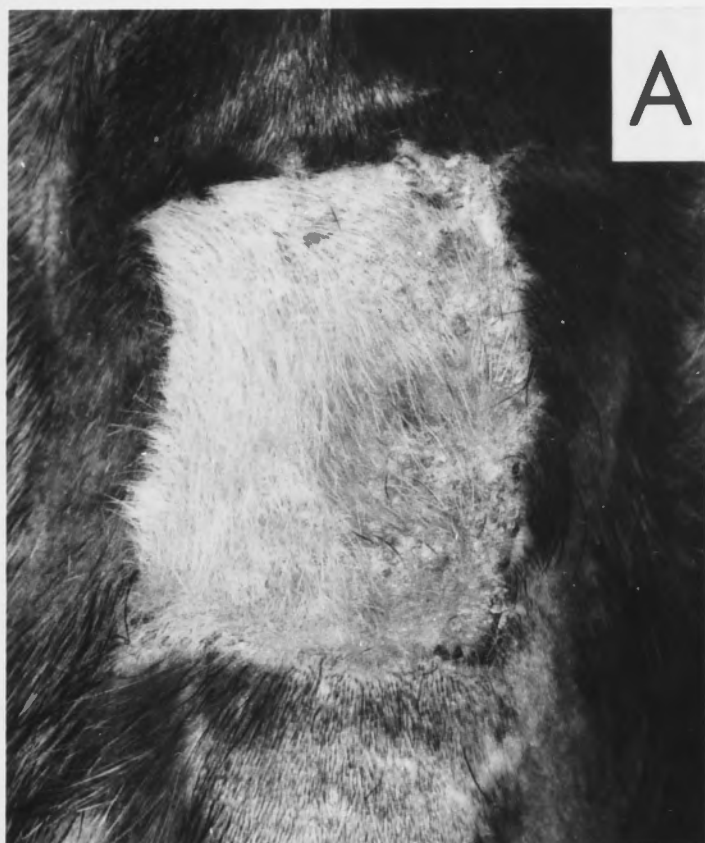
(A), (B) and (C) demonstrate the "rejection-recovery" episodes of the freshly placed (DA x Lewis) F_1 hybrid skin graft.

(A) At 22nd day - Desquamation was observed.

(B) At 30th day - Hard scab was observed. Hard scab was shed at 46th day and new fur started growing again at 50th day.

(C) At 65th day (15days after commencement of new fur growth) - New fur was growing well, although some necrotic lesions were still observed.

(a), (b) and (c) demonstrate a long-tolerated (DA x Lewis) F_1 hybrid skin graft (right) and a freshly placed (DA x Lewis) F_1 hybrid skin graft (left) at 22, 30 and 65 days after a fresh F_1 hybrid skin graft was placed and 4×10^8 Lewis thoracic duct lymphocytes were injected.



4) The fate of skin grafts on tolerant rats which have been injected with normal syngeneic lymphocytes following receipt of tolerated, allogeneic lymphocytes

The injection of specifically sensitized lymph node cells from syngeneic adult donors has been reported to abort graft-versus-host reactions previously initiated in neonatal rats by the transfer of allogeneic lymphocytes (Silvers and Billingham, 1969b). A group of experiments, based on this observation, were performed in homograft tolerant rats. Ten (DA x Lewis) F_1 hybrid-tolerant DA rats were injected intravenously with the indicated numbers of thoracic duct lymphocytes from normal Lewis rats and, subsequently, received an inoculum of lymphocytes from normal (non-sensitized) DA donors (Table 16). The fate of these rats and of their (DA x Lewis) F_1 hybrid skin grafts can be seen to fall into two distinct categories determined by the period that had elapsed between the two lymphocyte inocula. If the second injection (DA strain lymphocytes) was administered within three days of the first, the recipients remained quite healthy but their skin grafts were rapidly rejected. The course followed was indistinguishable from that observed to follow the injection of normal syngeneic lymphocytes alone (Section 4,A). In contrast, if the transfer of DA strain lymphocytes was deferred for four days or longer, the tolerant DA rats wasted and died from graft-versus-host disease. However, as was observed when similar rats received allogeneic lymphocytes alone (without any attempt to save them by the injection of syngeneic cells) their (DA x Lewis) F_1 hybrid skin grafts remained quite intact

TABLE 16

Effect of normal syngeneic T.D.L. on tolerated allogeneic T.D.L.-inoculated rats*.

Tolerant Rat No.	Primary injection ($\times 10^{-6}$)	Interval between primary & secondary injection	Secondary injection ($\times 10^{-6}$)	Fate of recipient Time of death or rejection after primary injection (days)
1 [‡]	400:DA	1	250:Lewis	rejected ⁺ (13)
2	400:Lewis	0	400:DA	rejected (12)
3	250:Lewis	1	400:DA	rejected (14)
4	250:Lewis	2	700:DA	rejected (15)
5 [•]	250:Lewis		Not given	died (16)
6	400:Lewis	3	400:DA	rejected (14)
7	400:Lewis	4	400:DA	died [§] (13)
8	400:Lewis	8	400:DA	died (16)
9	400:Lewis	8	300:DA	died (15)
10	400:Lewis	10	400:DA	died (15)

* All tolerant rats were DA strain rats tolerant of (DA x Lewis) F_1 hybrids.

+ All recipients which rejected tolerated- F_1 skin grafts survived without developing any signs of GvH disease.

• Rat No. 5 was injected with 2.5×10^8 Lewis T.D.L. alone.

‡ Rat No. 1 was injected with syngeneic DA T.D.L. first, followed by allogeneic Lewis T.D.L.

§ Long-established F_1 hybrid skin grafts on all recipients which died as a result of GvH disease remained intact.

at the time of death. It is interesting to note from Table 16 that the time required for the complete destruction of F_1 hybrid skin grafts in the rats which survived was slightly less than the time of survival of those rats which died with intact skin grafts.

G. The appearance of reactive cells in F_1 hybrid-tolerant rats following the injection of tolerated, allogeneic lymphocytes

The two preceding sections were concerned with the effects of transferring tolerated, allogeneic thoracic duct lymphocytes from normal donors to homograft tolerant recipients. It was observed that, with the two strains of rat examined, the transfer of 4×10^8 lymphocytes was invariably lethal unless the tolerant recipient had been previously injected with bone marrow cells from rats of the donor strain. Furthermore, it was found that the allogeneic lymphocytes injected, although capable of mounting a lethal graft-versus-host disease against the recipient, were not effective in mediating the rejection of tolerated skin grafts on that recipient. It was suggested that this latter observation might reflect the operation of some factor, such as an enhancing antibody, capable of protecting tolerated skin grafts from cell-mediated rejection. If this suggestion is valid, it would be predicted that cells with reactivity against the tissues of tolerated F_1 hybrid type should be demonstrable in tolerant rats following the injection of allogeneic lymphocytes.

To test this point (DA x Lewis) F_1 hybrid-tolerant rats of DA strain were intravenously injected with 4×10^8

tolerated, allogeneic (Lewis) thoracic duct lymphocytes and were themselves submitted to thoracic duct cannulation or splenectomy at various intervals afterwards. Cells obtained from these two sources were then transferred, in graded doses, to lightly irradiated (DA x Lewis) F_1 hybrid rats and the mortality of these recipients was followed. Figure 20 records the pattern of mortality observed in F_1 hybrid rats in receipt of thoracic duct lymphocytes (in dosage ranging from 5 to 160×10^6) collected from tolerant DA rats on the second to eleventh days after receipt of Lewis strain lymphocytes. While cells with anti- F_1 hybrid activity could not be detected in 160×10^6 thoracic duct lymphocytes collected on the second day, such cells appeared to comprise an appreciable fraction of the lymphocytes collected on the three following days. The death of F_1 hybrids injected with 20×10^6 lymphocytes in these experiments should be compared with the requirement for 5×10^6 lymphocytes from normal Lewis rats to uniformly kill similar F_1 hybrid rats (Section 3,A). Assessment of the anti- F_1 hybrid activity of thoracic duct lymphocytes collected at later times from injected, tolerant rats was complicated by a rapid decrease in the number of cells which could be collected from the fistulae. This lymphopenia was one feature of the graft-versus-host disease which first became apparent in the tolerant rats after eight days and then developed rapidly to cause death as early as thirteen days (Section 4,E). Despite the small numbers of circulating lymphocytes in such rats, it remained possible to demonstrate cells with anti- F_1 hybrid activity in the thoracic duct lymph of some rats

Fig. 20. G.v.H. activity of thoracic duct lymphocytes from DA rats tolerant of (DA x Lewis) F_1 hybrid tissues following their receipt of 4×10^8 normal Lewis thoracic duct lymphocytes.

		Time after injection of tolerated allogeneic T.D.L. (days)									
		2	3	4	5	6	7	8	9	10	11
No. of cells transferred ($\times 10^{-6}$)	160	△	▲			▲					
	80	△	▲ ▲	▲	▲	▲	▲		▲ ▲		
	40	△	△	▲	▲ ▲ △	△	▲	△	▲	△	△
	20	△	▲	△	▲ ▲	△	▲	△	▲	△	△
	10	△	△	▲	△ △	△	△	△	▲		△

G.v.H. activity of cells from inoculated tolerant rats was assessed by injection into irradiated (455 rads) (DA x Lewis) F_1 hybrid rats.

- ▲ (DA x Lewis) F_1 hybrid recipients which were killed within 50 days after injection of thoracic duct lymphocytes from inoculated tolerant rats.
- △ Recipients which were surviving 100 days after injection.

examined as late as nine days after injection of allogeneic lymphocytes.

Examination of the capacity of spleen cells from F_1 hybrid-tolerant DA rats, in receipt of lymphocytes from Lewis donors, to kill F_1 hybrid rats gave more consistent results (Figure 21). Comparison of the efficacy of cells from injected, tolerant DA donors with cells from the spleens of normal DA rats revealed that the former were the more lethal when collected from donors which had been injected 6 days, or less, previously. At longer intervals the capacity of spleen cells from tolerant donors to kill F_1 hybrids steadily decreased as the manifestations of graft-versus-host disease in the spleen donors became more florid.

Examination of the anti- F_1 hybrid activity of cells from both thoracic duct and spleen of F_1 hybrid-tolerant DA rats which had been transfused with Lewis strain lymphocytes in the preceding experiments indicated the presence of considerable numbers of such cells. In an attempt to define the origin of these reactive cells, lymphocytes from injected, tolerant DA rats were incubated with Lewis anti-DA serum or DA anti-Lewis serum before their anti- F_1 hybrid activity was measured by means of the popliteal lymph node assay of Ford, Burr and Simonsen (1970). As the number of such experiments performed was small, any conclusions must be tentative (Table 17). Nevertheless, it appears that, from 4 to 7 days after receipt of Lewis lymphocytes, the anti- F_1 hybrid activity of tolerant DA rats resides in Lewis strain cells. The results obtained at earlier periods are difficult to

Fig. 21. G.v.H. activity of spleen cells from DA rats tolerant of (DA x Lewis) F_1 hybrid tissues following the receipt of 4×10^8 normal Lewis thoracic duct lymphocytes.

		Time after injection of tolerated allogeneic T.D.L. (days)														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
No. of cells transferred ($\times 10^6$)	160		◆		◆◆		◆		◆		◆		◆			◆
	80		◆		◆◆		◆		◆		◆		◆		◆	◇
	40		◆*		◆◆		◆		◆		◆◆		◇		◇	◇
	20		◆◆		◆*		◆		◇		◇					

G.v.H. activity of cells from inoculated tolerant rats was assessed by injection into irradiated (455 rads) (DA x Lewis) F_1 hybrid rats.

- ◆ (DA x Lewis) F_1 hybrid recipients which were killed within 50 days¹ after injection of spleen cells from inoculated tolerant rats.
- ◆* Recipients which died between 50 and 70 days after injection.
- ◇ Recipients which were surviving 100 days after transfer of spleen cells.

TABLE 17

Origin of GvH reactive cells appearing in (DA x Lewis) F_1 -tolerant DA rats which received normal Lewis T.D.L. as determined by means of isoantiserum discrimination.

Time after injection	Cells measured for GvH	Popliteal lymph node weight [†] (mg) mean \pm standard error		
		Control*	Anti-DA	Anti-Lewis
(a)	4	14.4 \pm 2.2	10.0 \pm 1.1	10.7 \pm 0.9
	5	15.6 \pm 0.7	12.5 \pm 1.3	12.6 \pm 1.6
	6	14.3 \pm 1.5	12.5 \pm 1.4	5.0 \pm 0.5
(b)	4	16.7 \pm 1.1	30.3 \pm 1.8	9.3 \pm 0.9
(c)	7	12.5 \pm 0.5	12.3 \pm 1.0	5.1 \pm 0.4

The procedure for incubation was described in Section 2. 5×10^6 cells in 0.1 ml Hank's saline were injected into foot pads of (DA x Lewis) F_1 hybrid rats.

* Control represents injection of 5×10^6 unincubated cells.

+ Four popliteal lymph nodes of F_1 hybrid recipients were examined.

interpret and the presence of reactive cells of DA (host) origin cannot be excluded.

Support for the inferred donor origin of the anti- F_1 hybrid cells that appeared in F_1 hybrid-tolerant rats following the transfer of tolerated, allogeneic lymphocytes was provided by further experiments. It was not possible to demonstrate any anti-(DA x Lewis) F_1 hybrid activity in the spleen cells of F_1 hybrid-tolerant Lewis rats which had received Mitomycin C-treated DA strain lymphocytes or lymphocytes from donors of a third, unrelated (Hooded) strain. That is, allogeneic confrontation alone was not capable of evoking the reappearance of reactivity in homograft tolerant rats.

SECTION 5

Discussion of Results

A. The termination of homograft tolerance by means of the transfer of lymphocytes from normal, syngeneic donors

The pattern of reappearance of cells with graft-versus-host activity was examined in tolerant rats which had been injected with lymphocytes from normal syngeneic donors. A group of DA rats, tolerant of (DA x Lewis) F_1 hybrid tissues, each received an intravenous injection of 4×10^8 thoracic duct lymphocytes from normal DA donors. The pattern of reappearance of cells with reactivity against (DA x Lewis) F_1 hybrid tissues was examined by collecting cells from the thoracic duct or spleen of these injected, tolerant rats and transferring them to F_1 hybrids. Two types of assay were used to detect cells reactive against F_1 hybrid tissues.

In the first assay, a range of doses of the cells to be tested were transferred to F_1 hybrids which had been exposed to 455 rads of gamma irradiation. Death of the recipient was used as the end point. In the second assay, the ability of 10^7 cells to mount a local graft-versus-host reaction was examined by injecting cells into a foot-pad and measuring the extent of enlargement of the corresponding popliteal lymph node seven days later.

When the capacity of cells from tolerant rats injected with normal lymphocytes to kill sublethally irradiated F_1 hybrid rats was examined, thoracic duct

lymphocytes from such donors were found to be highly efficient as early as the third day after transfer. Thus, on the 3rd and 4th days, 4×10^7 lymphocytes from injected tolerant rats were uniformly lethal for F_1 hybrids (2×10^7 lymphocytes from normal donors were required for a similar effect). In contrast to this indication that a high degree of reactivity had rapidly returned, further examination of injected tolerant rats disclosed an abrupt decrease in the reactivity of circulating lymphocytes against F_1 hybrid tissues. This decrease in the reactivity of thoracic duct lymphocytes was most marked on the 6th and 7th days after injection of the tolerant donor. At this time, an eightfold increase over that dose of cells which was lethal if collected on the fourth day failed to kill F_1 hybrids. Reappearance of reactivity in thoracic duct lymphocytes was observed by the 9th day.

Examination of the reactivity of spleen cells from injected tolerant rats failed to reveal the biphasic pattern observed with thoracic duct lymphocytes. Although there was considerable variation between tolerant donors, the reactivity of spleen cells from injected tolerant rats between the 3rd and 10th day was at least equivalent to that of spleen cells from normal donors. Thus, an injection of 4×10^7 normal spleen cells proved lethal in 3 out of 9 F_1 hybrids while a similar inoculum from injected tolerant donors killed 13 out of 23. The two features of these experiments which require discussion are the very early appearance of a near-normal level of reactivity in lymphocytes collected from injected tolerant

rats and, subsequently, the marked absence of reactivity on the part of thoracic duct lymphocytes collected six or seven days after injection of a tolerant rat.

Although the rapidity of appearance of reactive cells after injection of a tolerant rat with normal lymphocytes was surprising, the ensuing disappearance of reactive cells was a more unexpected finding. It has been suggested that "selective-recruitment" of specific histocompatibility antigen-reactive cells can be effected if parental strain lymphocytes are injected into F_1 hybrid hosts (Ford and Atkins, 1971; Sprent and Miller, 1972). If this hypothesis is correct, it is conceivable that the present results reflect the selective trapping of specifically reactive cells from the parental strain inoculum in the chimaeric tissues of the tolerant host as a result of confrontation with F_1 hybrid strain cells. The reappearance of reactive cells on the 9th day would then be interpreted as the release of these sequestered cells. Examination of the reactivity of spleen cells was undertaken in an attempt to test this suggestion, as a corresponding increase in the splenic content of reactive cells might have been anticipated if the decrease in thoracic duct reactivity resulted from "selective trapping" in this organ.

The absence of any increase in splenic content of reactive cells indicates that any sequestration of such cells has not occurred in the spleen. As there is no indication given in the report of Ford and Atkins (1971) regarding the presence or absence of reactive cells in the spleen at the time

of disappearance of such cells from the thoracic duct (it was reported that reactivity of spleen cells of F_1 hybrid rats 12 hours after injection of parental strain thoracic duct lymphocytes did not differ significantly from that of normal parental strain lymphocytes), it is not possible to evaluate the present results in terms of other systems.

An alternative explanation for the lack of reactivity of thoracic duct lymphocytes from injected tolerant rats on the sixth and seventh days merits consideration. Elkins (1972) has reported that lymphoid cells from tolerant rats which have recently been injected with normal lymphoid cells can exert a suppressive effect on lymphoid cells from normal donors when assayed under the kidney capsule of F_1 hybrids. The suppressive effect was peculiar to cells from tolerant rats which had been injected with normal lymphocytes as lymphoid cells from uninjected tolerant donors lacked this activity (Elkins, 1972; Atkins and Ford, 1972). A similar capacity to suppress the reactivity of normal parental strain lymphocytes in the popliteal lymph node weight assay has been observed in parental strain lymphocytes which have been passaged through F_1 hybrids in the manner of Ford and Atkins (1971) (McCullagh, personal communication). Consequently, it is possible that cells with the ability to suppress the graft-versus-host activity of DA lymphocytes in irradiated (DA x Lewis) F_1 hybrids could be responsible for loss of activity of thoracic duct lymphocytes from injected tolerant rats.

To interpret the pattern of appearance of reactive

cells in tolerant rats that have been injected with normal lymphocytes, it is necessary to distinguish between reactive cells derived from the injected lymphocytes and those derived from precursors in the tolerant host. This distinction has been drawn by using thoracic duct lymphocytes from a donor of the opposite sex to the tolerant recipient. It was found that the graft-versus-host activity, at the time of its peak, in the circulating lymphocytes of the tolerant recipient, was attributable to donor type cells. In contrast, the activity appeared to be mediated by host cells in a rat examined 5 months after receipt of normal lymphocytes. If these results are generally applicable, it appears that "normal" lymphocytes transferred to a tolerant host rapidly appear as reactive cells in its circulation. However, within a week of transfer, such cells become infrequent and the reappearance of large numbers of reactive cells is delayed for a long period. This second return of reactivity occurs as a result of the generation of the appropriate cells from the lymphoid tissues of the host.

B. The termination of tolerance by means of cells passaged from "terminated-tolerant" donors

The results obtained in the present experiments should be compared with those of Silvers (1970) on the serial passage of spleen and lymph node cells. He found that none of the tertiary tolerant host mice (T_3) rejected skin homografts while only 1 out of 7 secondary hosts (T_2) and 7 of 10 primary tolerant hosts (T_1) rejected their tolerated grafts. The times required for rejection varied from 3 weeks to 4 months.

The present results differ in that graft rejection

occurred more rapidly and frequently and that the capacity to effect rejection could be passaged through more hosts. The use of thoracic duct lymphocytes rather than spleen and lymph node cells in the present experiments may be one of the reasons for this difference. Furthermore, it is probable that the numbers of cells passaged in the present experiments, relative to the size of the lymphoid tissues of the recipients, exceeded those used by Silvers, although comparisons between species are difficult.

The reduced graft-versus-host activity of thoracic duct lymphocytes with repeated passage could reflect the transfer of reduced numbers of reactive cells in successive passages. There may be a similar explanation for the prolonged rejection time observed with passages. If the re-equipment of a tolerant rat with appropriately reactive cells following the transfer of normal, syngeneic lymphocytes resulted from the proliferation of those transferred cells, it seems reasonable to predict that the capacity to terminate tolerance should have been passaged in the experiments. However, the experimental results presented in Section 4,A imply that the transferred normal cells do not effect this "re-equipment". Furthermore, the results in Sections 4C and D indicate that restoration of normal reactivity in tolerant rats requires the reappearance of reactive host cells. Taking account of these previous observations, the paucity of cells with capacity to terminate tolerance recovered in the present experiments may be attributable to the failure of any of these cells to be generated from the initial inoculum of normal thoracic duct

lymphocytes.

An alternative factor which should be considered in explaining the failure to passage cells with the ability to terminate tolerance is the appearance of suppressor cells (Elkins, 1972). Elkins has suggested that Silver's difficulty in passaging immunity in tolerant mice may have resulted from the presence of such cells. It is likely that both mechanisms, the dilution of reactive cells and the accumulation of "suppressor cells", could have been involved in interfering with the repeated termination of the tolerant state by passaged cells.

The graft-versus-host activity of lymphoid cells from all of the "terminated-tolerant" rats, including T_3 rats, were found to return to normal values, when measured at prolonged periods of time after receipt of lymphocytes as has been noted previously in Section 4,A in the case of tolerant recipients of normal syngeneic lymphocytes. The most striking feature of the experiments is the dichotomy which appears between rats in which a skin graft was rejected and those in which it survived. Comparison of tolerant rats in which grafts were rejected or retained after very similar treatment (namely the injection of comparable inocula of thoracic duct lymphocytes with graft-versus-host activity of the same order) reveals a complete return to normal levels of activity on the part of the lymphoid tissues of the former and a complete failure to regain any such activity in the latter group a year later. This observation cannot be reconciled with an explanation for failure of graft rejection based solely on the transfer of inadequate numbers of

reactive cells. If such an explanation was correct one might predict that graft rejection, although delayed, would eventually occur when the reactive cell population in the transferred inoculum had attained an appropriate size. Even if it was postulated that indefinite graft survival could occur in the face of a very gradual increase in the complement of reactive cells towards normal levels, it would be anticipated that the reactivity of the lymphoid cells in the injected, tolerant hosts would gradually approach normality. The conclusion is inescapable that some episode occurring shortly after the transfer of normal, syngeneic lymphocytes determines whether the lymphoid cells of a tolerant host are ultimately to return to normal reactivity or remain markedly impaired. The present experiments do not give any indication of the nature of this "all or nothing" event. The elimination of chimaerism could be suggested as a possibility. Experiments reported in Section 4,A in which the repeated injection of large numbers of bone marrow cells of the tolerated strain into tolerant rats failed to influence either graft rejection or recrudescence of cellular activity in any way does not support this suggestion. However, irrespective of the nature of the incident which determines whether a tolerant recipient of normal lymphocytes is or is not ultimately to regain normal reactivity, these results suggest that some positive influence antagonistic to the re-establishment of a normal reactivity exists in the tolerant animal.

The present results, while not excluding some elimination of the appropriate reactive cells as a basis for

homograft-tolerance are irreconcilable with an explanation for tolerance based solely on such an elimination.

C. Termination of homograft tolerance by means of antiserum

Previous observations of the pattern of reappearance of graft-versus-host reactivity following termination of the tolerant state by normal, syngeneic lymphocytes suggested that, whereas initial reactivity was attributable to the transferred cells, later competence resided in cells of host origin. The results of using F_1 hybrid animals pre-sensitized against the male specific antigen in which to assay reactivity also implied that fully reactive cells could be derived from the formerly-tolerant host. These conclusions were confirmed by examining the graft-versus-host reactivity of lymphoid cells from rats in which tolerance had been terminated by means of specific antiserum. The prolonged period required for graft rejection implies, as Lubaroff and Silvers (1970) suggested that antiserum was acting on the graft indirectly, possibly by eliminating the chimaeric state first. It was also noted, that, if antiserum treatment had failed to effect skin graft rejection, there was no reappearance of reactivity in the cells of the tolerant rat. This finding re-emphasizes the conclusion drawn from these experiments in which passage of thoracic duct lymphocytes failed to produce graft rejection in tolerant recipients, namely that some critical event, possibly elimination of the chimaeric state, is required before any reactive cells can be detected in tolerant rats.

D. The partial termination of homograft tolerance following the transfer of semi-allogeneic lymphocytes

A group of DA rats tolerant of (Hooded x Lewis) F_1

hybrid tissues was examined to test the hypothesis, formulated from earlier experiments on termination of homograft tolerance with normal, syngeneic lymphocytes, that the re-equipment of "terminated-tolerant" rats with specifically reactive lymphocytes is attributable to host type cells. These tolerant DA rats were injected with large numbers of thoracic duct lymphocytes collected from (DA x Lewis) F_1 hybrid donors specifically sensitized against Hooded-strain tissues. In response to this lymphocyte transfer, grafts of Hooded and (Lewis x Hooded) F_1 hybrid skins were rapidly rejected by all of the rats in the group whereas Lewis-strain skin grafts remained intact indefinitely. These observations on graft survival contrast with those reported by Silvers and Billingham (1970) following the transfer of lymphoid cells (one donor-equivalent of lymphoid cells) from either (Lewis x DA) F_1 rats sensitized against BN tissues, or (Lewis x BN) F_1 rats sensitized against DA tissues, to Lewis rats tolerant of (BN x DA) F_1 tissues. These workers observed that both BN strain and DA strain skin grafts were rejected in 16 out of 25 tolerant Lewis rats. The graft against which the transferred sensitivity was specifically directed was usually destroyed first, within eight days of lymphoid cell transfer, whereas rejection of the other graft occurred within 9-85 days, mostly within 50 days.

Subsequent observations of the DA rats used in the present experiments revealed that, over the course of five months, cells with reactivity directed against (DA x Hooded) F_1 hybrid tissues reappeared in the lymphoid tissues. Control experiments indicated that these cells were of DA type rather than of (DA x Lewis) F_1 hybrid type. That is, specific graft-

versus-host reactivity, although initially very reduced even after the termination of skin graft tolerance, reappeared in cells derived from the host. This is completely in accord with the observations previously made by use of the discriminative assay directed against male specific antigens. It is also subject to similar interpretations to those experiments in which homograft tolerance was terminated by means of iso-antiserum.

A second point raised for discussion by these experiments is the difference in fate of skin grafts in comparison with the experiments of Silvers and Billingham (1970). They observed that both BN and DA strain skin grafts were generally rejected by (BN x DA) F_1 -tolerant Lewis rats following the injection of lymphoid cells from BN-sensitized (Lewis x DA) F_1 rats or DA-sensitized (Lewis x BN) F_1 rats. They suggested that the transferred lymphoid cells were themselves responsible for the rejection of grafts against which the transferred sensitivity was specifically directed, whilst subsequent rejection of the other grafts was effected by reactive cells of host (Lewis) type which had reappeared after the elimination of chimaerism. It was inferred that lymph node cells were not a suitable source of cells for maintaining chimaerism when Ag-B incompatibilities are involved.

In the present experiments, although grafts of Hooded strain skin were rapidly rejected, Lewis strain skin grafts remained intact indefinitely. This might be taken to indicate either that the injected (DA x Lewis) F_1 hybrid thoracic duct lymphocytes had substituted for (Lewis x Hooded) F_1 hybrid type

cells in maintaining tolerance of Hooded strain tissues or that the transferred lymphocytes, while effecting rejection of Hooded skin grafts, failed to eliminate chimaerism. However, the later appearance of DA type cells reactive against Hooded tissues provides sound evidence for the earlier elimination of chimaeric (Lewis x Hooded) F_1 hybrid type cells. Hence it seems likely that the injected lymphocytes had successfully established a chimaeric state.

E. The production of graft-versus-host disease in homograft-tolerant rats and the modification of its course

Experiments involving the transfer of normal, syngeneic lymphocytes to homograft-tolerant rats do not lend themselves to analysis of the relative roles of the injected lymphocytes and the cells of the tolerant host in mediating rejection of previously-tolerated grafts. The injection of an inoculum of normal lymphocytes, allogeneic to but tolerated by, the tolerant host together with study of the fate of skin grafts of donor, host and (donor x host) F_1 hybrid type of a tolerant host could be more informative. The results from this type of experiment have been grouped, for convenience, into those observations concerning the fate of the tolerant recipients of allogeneic lymphocytes themselves and those concerning skin grafts on such rats.

1) Graft-versus-host disease following the transfer of tolerated, allogeneic thoracic duct lymphocytes to tolerant rats

The dose of tolerated, allogeneic lymphocytes used in these experiments (4×10^8) was uniformly lethal in the absence

of preliminary treatment of the tolerant recipient. In contrast to this result, the preliminary inoculation of 2×10^8 bone marrow cells from a Lewis donor was observed to protect 15 of 25 (DA x Lewis) F_1 hybrid-tolerant DA rats from death. However, bone marrow cells from DA rats were incapable, when used under similar conditions, of protecting (DA x Lewis) F_1 hybrid-tolerant Lewis rats from the lethal effects of DA strain lymphocytes. Several other observations may be relevant to the mechanism of the protection conferred by preliminary inocula. Thus, protection was most regularly conferred when tolerant female DA rats received bone marrow cells from male Lewis donors before being challenged with lymphocytes from male Lewis rats. Nevertheless, it was possible to protect tolerant male DA rats by means of preliminary inocula of bone marrow cells. Furthermore, examination of the protective effect of preliminary administration of Lewis-strain cells to (DA x Lewis) F_1 hybrids indicated that cells from female Lewis donors were effective in protecting female F_1 hybrids against the lethal effect of lymphocytes from male Lewis rats. In another group of experiments, Mitomycin C-treated lymphocytes from Lewis rats were shown to confer some protection against the subsequent injection of normal Lewis strain lymphocytes on tolerant DA rats. Finally, it was noted that DA rats tolerant of Lewis (as distinct from (DA x Lewis) F_1) tissues could be protected against the lethal effects of lymphocytes from Lewis rats by means of the injection of bone marrow cells from Lewis donors.

There appear to be several mechanisms by which the

preliminary injection of Lewis-strain bone marrow cells could have protected tolerant DA rats from a subsequent graft-versus-host reaction mounted by lymphocytes from Lewis donors. In the first place, the bone marrow inoculum could have supplied the tolerant host with stem cells which were not susceptible to attack by the thoracic duct lymphocytes which were subsequently injected. If deficiency of haemopoietic stem cells is a major factor in producing the clinical manifestations of graft-versus-host disease (Barnes, Loutit and Micklem, 1961), provision of a source of such cells resistant to attack would be expected to mitigate the manifestations of the syndrome. The observation that DA rats tolerant of Lewis tissues died after the injection of Lewis-strain lymphocytes but could be saved by a preliminary injection of Lewis-strain bone marrow cells does not invalidate this proposition. The very low frequency of chimaeric cells in the tissues of tolerant animals (Brent and Gowland, 1963b; Nakić, Mikuska, Kastelan, Springer and Silobrcić, 1970) might not provide sufficient stem cells of Lewis type, in the absence of supplementation, to confer protection against the manifestations of graft-versus-host disease.

A second possible explanation for the protective effect against graft-versus-host disease conferred by bone marrow cells is that this treatment induces an unresponsiveness in host lymphoid cells in regard to the proliferative response of these cells which provides the basis of a graft-versus-host reaction. The preliminary injection of Mitomycin C-treated lymphocytes of parental strain has been shown to protect F₁

hybrid rats against the lethal effects of untreated parental-strain lymphocytes (McCullagh, 1973). Attempts to protect tolerant DA rats by means of the injection of Mitomycin C-treated lymphocytes achieved some success in the present experiments, a result which could not be explained by the provision of stem cells. This raises the possibility that Lewis-strain bone marrow cells, which resemble Mitomycin C-treated lymphocytes in retaining antigenicity while lacking immune competence, protect tolerant DA rats from graft-versus-host disease by rendering their lymphoid cells unresponsive.

The third putative mechanism by which preliminary injection of bone marrow cells could confer protection against graft-versus-host disease is by the induction of immunity against male-specific antigens carried on the challenging dose of lymphocytes. That tolerant female DA rats were more readily protected by the injection of bone marrow cells than was the case with tolerant DA males supports this hypothesis. However, the fact that DA males could be protected, albeit less frequently, by the injection of Lewis-strain bone marrow precludes explanation of all cases of survival on the basis of immunization against male-specific antigen. The scope of this exclusion was extended by the demonstration that bone marrow cells from female donors could protect female hosts from the lethal effects of lymphocytes from male rats.

When these three hypotheses which seek to explain the protective effect of the preliminary injection of bone marrow cells are compared, the second suggestion, namely that the preliminary inoculum acts to render host lymphoid tissues

unresponsive to "attack" by allogeneic lymphocytes, is the only one to have general applicability. Nevertheless, it is possible that, at least in the case of some rats, the remaining two mechanisms may have contributed to survival.

2) The fate of skin grafts on homograft-tolerant rats following the transfer of tolerated, allogeneic lymphocytes

The experiments discussed in preceding sections (5,A and B) have been concerned with the fate of skin grafts on tolerant rats when the hosts have received inocula of normal, syngeneic lymphocytes. The results obtained have implied that, whereas rejection of a previously tolerated skin graft in this situation is mediated by the transferred cells, the re-equipment of the recipient with a normal degree of immunological reactivity entails the appearance of host-derived cells following termination of chimaerism. The experiments to be discussed in the present section were intended to clarify the role of transferred, normally reactive lymphocytes in the rejection of skin grafts by tolerant hosts.

Rats of either DA and Lewis strains which had been rendered tolerant of (DA x Lewis) F_1 hybrid tissues were injected with thoracic duct lymphocytes from donors of the other parental strain. The subsequent fate of skin grafts of DA, Lewis and F_1 hybrid type was followed as a means of assessing the origin of any cells capable of attacking the grafts. In a complementary group of experiments, the fate of similar skin grafts placed on normal F_1 hybrid rats was examined after transferring parental strain lymphocytes.

If F_1 hybrid-tolerant rats of DA or Lewis strain were

injected with 4×10^8 thoracic duct lymphocytes from a donor of the other strain death invariably occurred by 19 days as a result of graft-versus-host disease. However, the F_1 hybrid skin grafts borne by these rats remained in excellent condition at the time of death, microscopic examination failing to reveal other than a minimal increase in mononuclear cell content of the dermis. In contrast, long-standing F_1 hybrid skin grafts were completely rejected in shorter periods following the transfer of 4×10^8 syngeneic lymphocytes to a tolerant host. The death of tolerant recipients of allogeneic lymphocytes confirms that these cells were able not only to survive in their hosts but also to mount an effective attack against foreign tissues. Consequently, the failure of the transferred lymphocytes to destroy F_1 hybrid skin grafts in the time available before death of the host is puzzling. Two possible explanations were examined, namely that grafts of long-standing were more readily able to withstand attack than freshly placed skin and that engagement of the transferred lymphocytes by the tissues of the allogeneic host had precluded their mounting an attack on the skin grafts.

With regard to the former possibility, it is apparent from the rapidity with which long established skin grafts can be rejected following the transfer of normal syngeneic lymphocytes, that any increase in resistance on the part of such grafts, in comparison with freshly placed grafts, could only be minor. If grafts of (DA x Lewis) F_1 hybrid skin were freshly placed on tolerant rats shortly before the injection of allogeneic lymphocytes, there was some evidence of attack on

these grafts by the time of death of the host. While both the original and the freshly placed grafts were of normal appearance macroscopically, there were a few small foci of epithelial necrosis and some mononuclear infiltration in the fresh grafts although the tissue remained largely intact. These changes were more noticeable in tolerant Lewis rats in receipt of DA strain lymphocytes than in tolerant DA rats which had been injected with lymphocytes from Lewis donors. Although freshly placed grafts of F_1 hybrid type skin appeared to be more susceptible to attack than similar long established grafts, the extent of the damage produced in the former by the time of death of the tolerant hosts was certainly not sufficient to indicate that rejection would have occurred. By freshly applying grafts of skin from rats of each parental strain to these F_1 hybrid-tolerant rats, it was indicated that the changes observed in the grafts of F_1 hybrid skin were produced by the injected lymphocytes rather than by cells of host origin. Thus grafts syngeneic with the tolerant host showed microscopic changes similar to those in the F_1 hybrid grafts while skin grafts syngeneic with the transferred lymphocytes were completely free from these changes.

The second possibility, namely that allogeneic lymphocytes transferred to a tolerant rat become engaged in attacking host tissues to such an extent that skin grafts are not rejected before the death of the host was examined in (DA x Lewis) F_1 hybrids. The fate of skin grafts from donors of both parental strains placed on F_1 hybrids which were concurrently succumbing to graft-versus-host disease mounted by lymphocytes of one of

the parental strain has been studied by Billingham and Silvers (1961b). They found that all the skin grafts of different strain to the injected lymphocytes were rejected, while all grafts syngeneic with these lymphocytes survived. Similar experiments performed in the present study in which skin grafts of F_1 hybrids and the parental strain different to the injected lymphocytes were placed on F_1 hybrid rats revealed marked generalized necrosis in both types of skin grafts by the time that the F_1 hybrid hosts succumbed to graft-versus-host disease. As the time required for death was similar to that noted after the injection of tolerant rats with allogeneic lymphocytes, the striking difference in graft survival on the two types of hosts assumes considerable importance. It is possible to exclude the pathophysiology of graft-versus-host disease from responsibility for the relative resistance to rejection of F_1 hybrid grafts on tolerant rats. Furthermore, in view of the complete destruction of grafts on F_1 hybrid rats, it seems improbable that exclusive engagement of the transferred lymphocytes in attacking host tissues could account for the good condition of grafts on the tolerant hosts. The capacity of F_1 hybrid skin grafts to survive on tolerant hosts but not on F_1 hybrid hosts following the transfer of lymphocytes capable of recognizing them as "foreign" might be explained by the presence of some factor in the tolerant hosts capable of protecting against cellular attack. The presence in tolerant rats of such a factor - possibly an antigen-antibody complex - has been suggested by Bansal, Hellström, Hellström and Sjögren (1973). These workers observed that rats in which tolerance had been

induced as neonates and which had carried skin grafts for a prolonged period of time showed specifically directed blocking activity in their sera. This was detected by its effect on in vitro lymphocyte-mediated cytotoxicity.

The early curtailment of the present experiments by the death of the host following the transfer of allogeneic lymphocytes to tolerant rats makes it difficult to develop the preceding hypothesis. This shortcoming was avoided in a group of experiments in which tolerant rats were protected from the graft-versus-host disease that follows the injection of tolerated, allogeneic lymphocytes by the previous transfer of bone marrow cells. The mechanism of the protection afforded by bone marrow cells in this situation has been discussed in Section 5E-1. The uniform experience in all ten experiments of this type was that freshly-placed grafts of F_1 hybrid skin survived indefinitely. The occurrence of transient changes, unmistakably manifestations of a homograft reaction, noted in four of these rats indicated continuing activity on the part of the injected lymphocytes as late as four weeks after their transfer. It seems likely that the histological changes previously observed in F_1 hybrid grafts at the time of death of their tolerant hosts represented such reversible episodes of rejection.

Demonstration that F_1 hybrid skin grafts can survive indefinitely on F_1 hybrid-tolerant hosts despite the injection of large numbers of normal lymphocytes which should be capable of destroying them may be explicable on the basis of the presence of enhancing antibodies in the tolerant hosts. Such antibodies, directed against the tolerated F_1 hybrid tissues, would be likely to cover the surface both of cells in the F_1 hybrid skin

grafts and of the injected lymphocytes collected from donors of the other parental strain. In contrast, normal lymphocytes syngeneic with the tolerant host would not be coated in this way after transfer.

In a final group of experiments, the response of tolerant rats, and their skin grafts, to the injection of both tolerated allogeneic and normal syngeneic lymphocytes was examined. The results were quite well defined. Provided syngeneic lymphocytes were transferred sufficiently soon after the allogeneic inoculum, the development of graft-versus-host disease could be averted. This is in accordance with the observation of Silvers and Billingham (1969b), although the use of specifically sensitized syngeneic cells was found to be essential in their experiments. Survival of a tolerant rat was accompanied by the rapid destruction and sloughing of its skin graft. In contrast, if normal syngeneic lymphocytes were transferred too late to avert graft-versus-host disease, the tolerant recipients died with intact grafts of F_1 hybrid skin. This clear distinction in the fate of rats and skin grafts recalls the earlier observations of the injection of syngeneic and allogeneic lymphocytes alone.

F. The appearance of reactive cells in F_1 hybrid-tolerant rats following the injection of tolerated, allogeneic lymphocytes

It was suggested in the preceding section that failure of rejection of F_1 hybrid skin grafts by tolerant parental strain rats after receipt of lymphocytes from donors of the other parental strain might reflect the activity of enhancing antibodies. If this were so, cells with activity against F_1

hybrid tissues should remain detectable in these tolerant hosts. When cells with the capacity to kill F_1 hybrids were sought, large numbers could be demonstrated in both thoracic duct lymph and spleen of injected tolerant rats. Examination of the origin of these reactive cells, while not excluding the presence of some cells of host type, indicated that many were derived from the lymphocytes injected into the tolerant host.

This demonstration that cells reactive against F_1 hybrid tissues were present in tolerant rats following the injection of tolerated, allogeneic lymphocytes would have been predicted in view of the previous observation that such recipients succumb to graft-versus-host disease. It has some additional interest when considered in association with the earlier conclusion that appreciable numbers of reactive cells of host origin do not reappear for some months after the transfer of normal, syngeneic lymphocytes to tolerant rats. If normally reactive host cells had constituted a large fraction of those cells with anti- F_1 hybrid activity in the present experiments, their existence should have been detected by the use of iso-antisera.

G. General discussion

The experiments presented in this thesis have a bearing on three aspects of the cellular basis of homograft tolerance.

1) The activity of normally reactive lymphocytes following their transfer to tolerant hosts

The present results are completely inconsistent with a role for the transferred, normal lymphocytes as the

progenitors of cells which re-equip the formerly tolerant host with the reactivities that it lacked. It appears that the role of these transferred normal cells does not extend beyond the interruption of those influences which are responsible for maintaining the tolerant state. The fate of the transferred lymphocytes after the tolerant state has been terminated is more doubtful. While their failure to contribute to the ultimate reactive cell population of the "terminated-tolerant" animal implies that they are unable to replicate throughout its lymphoid tissues, it does not necessarily imply that they have failed to survive.

The means by which transferred, normal lymphocytes interrupt the processes responsible for the maintenance of the tolerant state remain speculative. It does appear certain that termination of the tolerant state is an event the occurrence of which is determined at an early stage after lymphocyte transfer. Thus, the transfer of an inoculum of lymphocytes capable of terminating tolerance is followed by the slow reappearance of reactive cells derived from the host whereas transfer of a slightly suboptimal inoculum fails to influence the tolerant state. One does not observe a more retarded appearance of reactive host cells in the latter case such as might have been predicted if the transferred lymphocyte population had been able to expand to a level at which it could terminate tolerance.

The visible parameter of termination of tolerance following normal lymphocyte transfer is the rejection of a skin homograft. It is clear that, in the absence of graft rejection, there is no reappearance of reactive cells in the lymphoid

tissues of a tolerant recipient of normal lymphocytes. It is suggested that, coincident with skin graft rejection, the elimination of other chimaeric cells from the tolerant animal may be the major determinant of the subsequent re-appearance of reactive cells.

One paradox concerning the later activity of transferred normal lymphocytes in tolerant hosts arises from the rapid rejection by "terminated-tolerant" rats of subsequent skin grafts. Whereas such rats remain markedly deficient in reactive cells as assessed by their capacity to mount graft-versus-host reactions for a prolonged period, they are simultaneously able to destroy fresh skin grafts with a tempo comparable to that of a second set rejection. It is difficult to reconcile these conflicting observations in any way other than accepting that the responsibility for graft-versus-host type reactions and homograft rejection is not shared by a common cell type. If this is so, it is conceivable that normal lymphocytes that have been transferred to a tolerant host may adoptively transfer the capacity to reject skin grafts for some time while failing to contribute a supply of cells able to undertake graft-versus-host reactions.

2) The re-equipment of "terminated-tolerant" rats with reactive cells

The present experiments are applicable only to the subject of graft-versus-host reactive cells, as no attempt was made to quantitate the reappearance of, or to identify the source of, cells capable of effecting homograft rejection. If the suggestion advanced in the preceding discussion, namely that

one cell type may not mediate both activities, is correct then the scope of the present discussion should be limited accordingly. With this provision, the present experiments indicate that the reactive cells which appear in "terminated-tolerant" rats are derived exclusively from the cells of the host. A similar conclusion has been drawn recently by Elkins (1973). Furthermore, the extended period which was required for the reappearance of these cells was more consistent with their generation by the expansion of a small number of precursors, than with their recruitment as a result of the reactivation of reversibly suppressed cells. Elkins' demonstration that thymectomy of "terminated-tolerant" rats interfered with the reappearance of reactivity supports this contention. These observations cannot exclude the existence of suppressed cells in homograft tolerant rats. However, they fail to support the proposition that the cessation of such suppression is a feature of the return of these animals to immunological normality.

3) The significance of factors actively encouraging the survival of homografts in tolerant rats

As discussed in Section 1, there is experimental support for the suggestion that the survival of skin homografts on tolerant hosts may be a consequence of specific serum "blocking" factors, possibly enhancing antibodies. The present experiments although not concerned with the direct investigation of such speculative mechanism, produced some results which could be interpreted on the basis of an active response, humoral or cellular, facilitating the survival of grafts on tolerant hosts.

The abrupt "disappearance" of reactive cells from the thoracic duct of "terminated-tolerant" rats late in the first week is more likely to have been a reflection of the presence of active suppressor cells as detected in a similar situation by Elkins (1972) than a manifestation of the "selective-recruitment" of Ford and Atkins (1971) there being no evidence of sequestration of reactive cells in the spleen. It seems feasible that the appearance of such suppressor cells in tolerant recipients of normal lymphocytes might explain the difficulty experienced in attempting to passage such cells through tolerant hosts. To concede that cells with suppressor activity may exist in tolerant animals is not to postulate that these cells are the major factor in maintenance of tolerance. Indeed, many of the results obtained in these experiments imply that they are not.

Another group of observations made following the transfer of tolerated, fully allogeneic lymphocytes to tolerant rats, are difficult to reconcile with an explanation of tolerance based solely on unreactivity of such animals to tolerated tissues. That the responsiveness of tolerated rats to tolerated lymphocytes, as indicated by their liability to respond in a graft-versus-host response, can be modified implies that some recognition of tolerated cells does occur. This may be analogous to the complete prevention of graft-versus-host disease that follows the transfer of normal, parental strain lymphocytes to F₁ hybrid rats which have been exposed to such cells previously (McCullagh, 1973). In both cases, it is inferred that because the propensity of the host to respond with the expected pathological features can be curtailed by previous treatment of the

host, its role in the reaction cannot be entirely passive. Finally, the behaviour of skin homografts on tolerant recipients of tolerated, allogeneic lymphocytes should be cited. Comparison of the fate of F_1 hybrid strain grafts on F_1 hybrid hosts and on F_1 hybrid-tolerant hosts of parental strain after lymphocyte injection reveals that, whereas the former are successfully rejected, the latter survive. This implies that the factors encouraging the survival of F_1 hybrid strain skin grafts are much stronger on a host that is artificially tolerant of this tissue than in a host which displays self-tolerance towards the graft. Similarly the episodic rejection/recovery course of fresh grafts on tolerant rats in receipt of allogeneic lymphocytes strongly suggests that some activity tending to prolong graft survival is operative. Unfortunately, the observations do not give any indication of the nature of this activity.

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